Method for obtaining a pathogen resistance in plants

Description

The invention relates to methods for generating or increasing a pathogen resistance in plants by reducing the expression, activity or function of an NADPH oxidase.

The aim of plant biotechnology work is the generation of plants with advantageous novel properties, for example for increasing agricultural productivity. The plants' natural defense mechanisms against pathogens are frequently insufficient. Fungal diseases alone result in annual yield losses of many billions of US$. The introduction of foreign genes from plants, animals or microbial sources can increase the defenses. Examples are the protection against feeding damage by insects by expressing *Bacillus thuringiensis* endotoxins (Vaeck et al. (1987) Nature 328:33-37) or the protection against fungal infection by expressing a bean chitinase (Broglie et al. (1991) Science 254:1194-1197). However, most of the approaches described only offer resistance to a single pathogen or a narrow spectrum of pathogens.

Only a few approaches exist which impart a resistance to a broader spectrum of pathogens to plants. Systemic acquired resistance (SAR) - a defense mechanism in a variety of plant/pathogen interactions - can be conferred by the application of endogenous messenger substances such as jasmonic acid (JA) or salicylic acid (SA) (Ward, et al. (1991) Plant Cell 3:1085-1094; Uknes, et al. (1992) Plant Cell 4(6):645-656). Similar effects can also be achieved by synthetic compounds such as 2,6-dichloroisonicotinic acid (INA) or S-methyl benzo(1,2,3)thiadiazole-7-thiocarboxylate (BTH; Bion®) (Friedrich et al. (1996) Plant J 10(1):61-70; Lawton et al. (1996) Plant J. 10:71-82). The expression of pathogenesis-related (PR) proteins, which are upregulated in the case of SAR, may also cause pathogen resistance in some cases.

In barley, the Mlo locus has been described as a negative regulator of the defense against pathogens. The loss of the Mlo gene causes an increased and, above all, race-unspecific resistance against a large number of mildew species (Büschges R et al. (1997) Cell 88:695-705; Jorgensen JH (1977) Euphytica 26:55-62; Lyngkjaer MF et al. (1995) Plant Pathol 44:786-790). Mlo-deficient barley varieties obtained by conventional breeding are already being used in agriculture. Despite intensive cultivation, the resistance has proved to be durable, presumably due to the fact that it is recessive. Mlo-like resistances in other plants,

in particular in cereal species, are not described. The Mlo gene and various homologs from other cereal species have been identified and cloned (Büschges R et al. (1997) Cell 88:695-705; WO 98/04586; Schulze-Lefert P, Vogel J (2000) Trends Plant Sci. 5:343-348). Various methods using these genes for obtaining a pathogen resistance are described (WO 98/04586; WO 00/01722; WO 99/47552). The disadvantage is that the Mlo-mediated defense mechanism comprises a spontaneous die-off of leaf cells (Wolter M et al. (1993) Mol Gen Genet 239:122-128). Another disadvantage is that the Mlo-deficient genotypes show hypersensitivity to hemibiotrophic pathogens such as *Magnaporte grisea* (*M. grisea*) and *Cochliobolus sativus* (*Bipolaris sorokiniana*) (Jarosch B et al. (1999) Mol Plant Microbe Interact 12:508-514; Kumar J et al. (2001) Phytopathology 91:127-133).

The liberation of reactive oxygen species (ROS; for example superoxide (O_2-), hydroxyl radicals and H_2O_2) is ascribed an important protection function in the reaction on plant pathogens (Wojtaszek P (1997) Biochem J 322:681-692). A variety of ways of how a cell can produce ROS are known. In the macrophages of mammals, it is in particular the enzyme NADPH oxidase, which is able to transfer electrons to molecular oxygen, which must be mentioned. Homologous enzymes have also been identified in plants (Lamb & Dixon (1997) Annu Rev Plant Physiol Plant Mol Biol 48:251).

It has been shown that mutations in the catalytic subunit of NADPH oxidase in Arabidopsis thaliana show a reduced accumulation of reactive oxygen intermediates (ROI). With regard to the hypersensitive reaction (HR), the results were heterogeneous: while infection with the aviralulent and bactrium Pseudomonas syringae showed a reduced HR in a double mutant, the virulent oomycete Peronospora parasitica showed an increased HR. Growth - both of virulent and of avirulent P. syringae strains - was not changed in comparison with wild-type plants, however (Torres MA et al. (2002) Proc Natl Acad Sci USA 99:517-522). Likewise, the inhibition of NADPH oxidase by means of the inhibitor diphenyleneiodonium chloride (DPI) - at physiologically relevant concentrations - had no effect on the development of pathogenic fungi (Hückelhoven R & Kogel KH (1998) Mol Plant Microbe Interact 11:292-300). A cDNA fragment of a phagocytic barley NADPH oxidase (*pNAox*, homolog of the large subunit *gp91phox* of a phagocytic NADPH oxidase) is described under the GenBank Acc.-No.: AJ251717).

The present invention aims at providing novel compounds for the defense against pathogens in plants, which compounds bring about an efficient defense against as broad as possible a pathogen spectrum in as many different plant species as possible, prefer-

ably the crop plants used in agriculture. We have found that this object is achieved by the present method.

A first aspect of the invention comprises a method for generating or increasing the resistance to at least one pathogen in plants, which comprises the following operating steps:

a) reduction of the protein quantity, activity or function of an NADPH oxidase in a plant or a tissue, organ, part or cell thereof, and

b) selection of the plants in which - in contrast or in comparison with the starting plant - the resistance to at least one pathogen exists or is increased.

Surprisingly, the reduction of the expression of a barley NADPH oxidase (pNAox) in the epidermal cell by a sequence-specific RNA interference approach using double-stranded pNAox-dsRNA ("gene silencing") shows a significantly reduced disease level following *Bgh* infection (measured with reference to the formulation of Haustoria). This finding is particularly surprising because the release of reactive oxygen species ("oxidative burst"), which is associated with NADPH oxidase, is generally ascribed a protective function.

Similar to Mlo, the reduction of the NADPH oxidase expression mediates a broad resistance to various isolates of Blumeria graminis f.sp. hordei. In transient gene silencing experiments, the penetration efficiency (development of Haustoria) of Bgh is reduced significantly by more than 35% - an effect which, in its intensity, corresponds to the effect obtained by means of Mlo-dsRNA (Schweizer P et al. (2000) Plant J 24:895-903). In the wild-type barley variety Pallas, approximately 40% of the fungal penetrations result in the development of haustoria, while the penetration rate in the case of reduced NADPH oxidase expression by introduction of a double-stranded RNA of NADPH oxidase (pNAox-dsRNA) only amounts to approximately 25%. The fact that even in pathogen-sensitive wild-type varieties such as Pallas only a penetration rate of approximately 40 to 50% can be observed can be attributed to the basal resistance, which is always present. Owing to these findings, the enzyme NADPH oxidase can be considered a key element for the successful penetration of a pathogen such as Bgh into the plant cell. In addition, the method is superior to all those methods where a pathogen-resistant phenotype is generated by overexpression of a resistance-mediating protein. Switching off a gene can be done without expression of a (foreign) protein. In the ideal case, only the endogenous gene is de-

activated. This has not inconsiderable advantages regarding approval and acceptance by the consumer, who is frequently unsure about plants with foreign proteins. Very especially advantageous in this context is the use of inducible promoters for reducing the NADPH oxidase quantity, activity or function, which, for example in the case of pathogen-inducible promoters, makes possible an expression only when required (i.e. attack by pathogens).

In principle, the method according to the invention can be applied to all plant species, preferably to those in which an NADPH oxidase or a functional equivalent thereof is expressed naturally.

For the purposes of the invention, "plant" means all genera and species of higher and lower plants of the Plant Kingdom. Included in this expression are the mature plants, seed, shoots and seedlings, and parts, propagation material, plant organs, tissues, protoplasts, callus and other cultures, for example cell cultures, derived from them, and all other types of groups of plant cells which give functional or structural units. Mature plants refers to plants at any developmental stage beyond that of the seedling. Seedling means a young, immature plant in an early developmental stage. "Plant" comprises all annual and perennial monocotyledonous and dicotyledonous plants and includes by way of example, but not by limitation, those of the genera Cucurbita, Rosa, Vitis, Juglans, Fragaria, Lotus, Medicago, Onobrychis, Trifolium, Trigonella, Vigna, Citrus, Linum, Geranium, Manihot, Daucus, Arabidopsis, Brassica, Raphanus, Sinapis, Atropa, Capsicum, Datura, Hyoscyamus, Lycopersicon, Nicotiana, Solarium, Petunia, Digitalis, Majorana, Cichorium, Helianthus, Lactuca, Bromus, Asparagus, Antirrhinum, Heterocallis, Nemesis, Pelargonium, Panieum, Pennisetum, Ranunculus, Senecio, Salpiglossis, Cucumis, Browaalia, Glycine, Pisum, Phaseolus, Lolium, Oryza, Zea, Avena, Hordeum, Secale, Triticum, Sorghum, Picea and Populus.

The term "plant" preferably comprises monocotyledonous crop plants, such as, for example, cereal species such as wheat, barley, millet, rye, triticale, maize, rice, sorghum or oats, and sugar cane.

The term furthermore comprises dicotyledonous crop plants such as, for example

- Brassicaceae such as oilseed rape, canola, cress, Arabidopsis, cabbages or canola, Leguminosae such as soybean, alfalfa, pea, beans or peanut

- Solanaceae such as potato, tobacco, tomato, egg plant or paprika, Asteraceae such as sunflower, Tagetes, lettice or Calendula,

- Cucurbitaceae such as melon, pumpkin/squash or zucchini,

and linseed, cotton, hemp, clover, spinach, flax, red pepper, carrot, beet, radish, sugar beet, sweet potato, cucumber, chicory, cauliflower, broccoli, asparagus, onion, garlic, celeriac, strawberry, raspberry, blackberry, pineapple, avocado, and the various tree, bush, nut and vine species. Tree species preferably comprises plum, cherry, peach, nectarine, apricot, banana, paw paw, mango, apple, pear, quince.

Furthermore comprised are ornamental plants, useful or ornamental trees, flowers, cut flowers, shrubs or lawn, by way of example but not by way of limitation, the families of the Rosaceae such as rose, Ericaceae such as rhododendrons and azaleas, Euphorbiaceae such as poinsettias and croton, Caryophyllaceae such as carnations, Solanaceae such as petunias, Gesneriaceae such as African violets, Balsaminaceae such as touch-me-not, Orchidaceae such as orchids, Iridaceae such as gladioli, iris, freesia and crocus, Compositae such as calendula, Geraniaceae such as geraniums, Liliaceae such as dracaena, Moraceae such as ficus, Araceae such as philodendron, and many others.

Preferred for the purposes of the invention are those plants which are employed as food or feedstuff, very especially preferably monocotyledonous genera and species, such as the above-described cereal species.

The method is very especially preferably applied to monocotyledonous plants, most preferably to plants with agricultural importance such as wheat, oats, millet, barley, rye, maize, rice, buckwheat, sorghum, triticale, spelt, linseed or sugar cane.

"Pathogen resistance" denotes the reduction or weakening of disease symptoms of a plant following infection by a pathogen. The symptoms can be manifold, but preferably comprise those which directly or indirectly have an adverse effect on the quality of the plant, the quantity of the yield, the suitability for use as feedstuff or foodstuff, or else which make sowing, planting, harvesting or processing of the crop difficult.

"Conferring", "existing", "generating" or "increasing" a pathogen resistance means that the defense mechanisms of a specific plant species or variety is increasingly resistant to one or more

pathogens due to the use of the method according to the invention in comparison with the wild type of the plant ("original plant"), to which the method according to the invention has not been applied, under otherwise identical conditions (such as, for example, climatic conditions, growing conditions, pathogen species and the like). The increased resistance manifests itself preferably in a reduced manifestation of the disease symptoms, disease symptoms comprising - in addition to the abovementioned adverse effects - for example also the penetration efficiency of a pathogen into the plant or plant cells or the proliferation efficiency in or on the same. In this context, the disease symptoms are preferably reduced by at least 10% or at least 20%, especially preferably by at least 40% or 60%, very especially preferably by at least 70% or 80% and most preferably by at least 90% or 95%.

"Selection" with regard to plants in which - as opposed or as compared to the original plant - resistance to at least one pathogen exists or is increased means all those methods which are suitable for recognizing an existing or increased resistance to pathogens. These may be symptoms of pathogen infection (for example the development of haustoria in the case of fungal infection), but may also comprise the above-described symptoms which relate to the quality of the plant, the quantity of the yield, the suitability for use as feedstuff or foodstuff and the like.

"Pathogen" within the scope of the invention means by way of example but not by limitation viruses or viroids, bacteria, fungi, animal pests such as, for example, insects or nematodes. Especially preferred are fungi, such as mildew. However, it can be assumed that the expression of an NADPH oxidase, its activity or its function also brings about resistance to other pathogens. The following pathogens may be mentioned by way of example but not by limitation:

1. Fungal pathogens and fungus-like pathogens:

   Fungal pathogens and fungus-like pathogens (such as, for example, Chromista) are preferably from the group comprising Plasmodiophoramycota, Oomycota, Ascomycota, Chytridiomycetes, Zygomycetes, Basidiomycota and Deuteromycetes (Fungi imperfecti). The pathogens mentioned in Tables 1 and 2 and the diseases with which they are associated may be mentioned by way of example but not by limitation.

Table 1: Fungal plant diseases

Disease	Pathogen
Leaf ruse	Puccinia recondita
Yellow rust	P. striiformis
Powdery mildew	Erysiphe graminis / Blumeria graminis
Glume blotch	Septoria nodorum
Leaf blotch	Septoria tritici
Ear fusarioses	Fusarium spp.
Eyespot	Pseudocercosporella herpotrichoides
Smut	Ustilago spp.
Bunt	Tilletia caries
Take-all	Gaeumannomyces graminis
Anthrocnose leaf blight Anthracnose stalk rot	Colletotrichum graminicola (teleomorph: Glomerella graminicola Politis); Glomerella tucumanensis (anamorph: Glomerella falcatum Went)
Aspergillus ear and kernel rot	Aspergillus flavus
Banded leaf and sheath spot	Rhizoctonia solani Kuhn = Rhizoctonia microsclerotia J. Matz (telomorph: Thanatephorus cucumeris)
Black bundle disease	Acremonium strictum W. Gams = Cephalosporium acremonium Auct. non Corda
Black kernel rot	Lasiodiplodia theobromae = Botryodiplodia theobromae
Borde blanco	Marasmiellus sp.
Brown spot (black spot, stalk rot)	Physoderma maydis
Cephalosporium kernel rot	Acremonium strictum = Cephalosporium acremonium
Charcoal rot	Macrophomina phaseolina
Corticium ear rot	Thanatephorus cucumeris = Corticium sasakii
Curvularia leaf spot	Curvularia clavata, C. eragrostidis = C. maculans (teleomorph: Cochliobolus eragrostidis), Curvularia inaequalis, C. intermedia (teleomorph: Cochliobolus intermedius), Curvularia lunata (teleomorph: Cochliobolus lunatus), Curvularia pallescens (teleomorph: Cochliobolus pallescens), Curvularia senegalensis, C. tuberculata (teleomorph: Cochliobolus tuberculatus)
Didymella leaf spot	Didymella exitalis

Disease	Pathogen
Diplodia ear rot and stalk rot	Diplodia frumenti (teleomorph: Botryosphaeria festucae)
Diplodia ear rot, stalk rot, seed rot and seedling blight	Diplodia maydis = Stenocarpella maydis
Diplodia leaf spot or streak	Stenocarpella macrospora = Diplodialeaf macrospora

Table 2: Downy mildew

Disease	Pathogen
Brown stripe downy mildew	Sclerophthora rayssiae var. zeae
Crazy top downy mildew	Sclerophthora macrospora = Sclerospora macrospora
Green ear downy mildew (graminicola downy mildew)	Sclerospora graminicola
Java downy mildew	Peronosclerospora maydis = Sclerospora maydis
Philippine downy mildew	Peronosclerospora philippinensis = Sclerospora philippinensis
Sorghum downy mildew	Peronosclerospora sorghi = Sclerospora sorghi
Spontaneum downy mildew	Peronosclerospora spontanea = Sclerospora spontanea
Sugarcane downy mildew	Peronosclerospora sacchari = Sclerospora sacchari
Dry ear rot (cob, kernel and stalk rot)	Nigrospora oryzae (teleomorph: Khuskia oryzae)
Ear rots, minor	Alternaria alternata = A. tenuis, Aspergillus glaucus, A. niger, Aspergillus spp., Botrytis cinerea (teleomorph: Botryotinia fuckeliana), Cunninghamella sp., Curvularia pallescens, Doratomyces stemonitis = Cephalotrichum stemonitis, Fusarium culmorum, Gonatobotrys simplex, Pithomyces maydicus, Rhizopus microsporus Tiegh., R. stolonifer = R. nigricans, Scopulariopsis brumptii
Ergot (horse's tooth)	Claviceps gigantea (anamorph: Sphacelia sp.)
Eyespot	Aureobasidium zeae = Kabatiella zeae

Disease	Pathogen
Fusarium ear and stalk rot	Fusarium subglutinans = F. moniliforme var.subglutinans
Fusarium kernel, root and stalk rot, seed rot and seedling blight	Fusarium moniliforme (teleomorph: Gibberella fujikuroi)
Fusarium stalk rot, seedling root rot	Fusarium avenaceum (teleomorph: Gibberella avenacea)
Gibberella ear and stalk rot	Gibberella zeae (anamorph: Fusarium graminearum)
Gray ear rot	Botryosphaeria zeae = Physalospora zeae (anamorph: Macrophoma zeae)
Gray leaf spot (Cercospora leaf spot)	Cercospora sorghi = C. sorghi var. maydis, C. zeae-maydis
Helminthosporium root rot	Exserohilum pedicellatum = Helminthosporium pedicellatum (teleomorph: Setosphaeria pedicellata)
Hormodendrum ear rot (Cladosporium rot)	Cladosporium cladosporioides = Hormodendrum cladosporioides, C. herbarum (teleomorph: Mycosphaerella tassiana)
Hyalothyridium leaf spot	Hyalothyridium maydis
Late wilt	Cephalosporium maydis
Leaf spots, minor	Alternaria alternata, Ascochyta maydis, A. tritici, A. zeicola, Bipolaris victoriae = Helminthosporium victoriae (teleomorph: Cochliobolus victoriae), C. sativus (anamorph: Bipolaris sorokiniana = H. sorokinianum = H. sativum), Epicoccum nigrum, Exserohilum prolatum = Drechslera prolata (teleomorph: Setosphaeria prolata) Graphium penicillioides, Leptosphaeria maydis, Leptothyrium zeae, Ophiosphaerella herpotricha, (anamorph: Scolecosporiella sp.), Paraphaeosphaeria michotii, Phoma sp., Septoria zeae, S. zeicola, S. zeina
Northern corn leaf blight (white blast, crown stalk rot, stripe)	Setosphaeria turcica (anamorph: Exserohilum turcicum = Helminthosporium turcicum)
Northern corn leaf spot Helminthosporium ear rot (race 1)	Cochliobolus carbonum (anamorph: Bipolaris zeicola = Helminthosporium carbonum)
Penicillium ear rot (blue eye, blue mold)	Penicillium spp., P. chrysogenum, P. expansum, P. oxalicum
Phaeocytostroma stalk rot and root rot	Phaeocytostroma ambiguum, = Phaeocytosporella zeae

Disease	Pathogen
Phaeosphaeria leaf spot	Phaeosphaeria maydis = Sphaerulina maydis
Physalospora ear rot (Botryosphaeria ear rot)	Botryosphaeria festucae = Physalospora zeicola (anamorph: Diplodia frumenti)
Purple leaf sheath	Hemiparasitic bacteria and fungi
Pyrenochaeta stalk rot and root rot	Phoma terrestris = Pyrenochaeta terrestris
Pythium root rot	Pythium spp., P. arrhenomanes, P. graminicola
Pythium stalk rot	Pythium aphanidermatum = P. butleri L.
Red kernel disease (ear mold, leaf and seed rot)	Epicoccum nigrum
Rhizoctonia ear rot (sclerotial rot)	Rhizoctonia zeae (teleomorph: Waitea circinata)
Rhizoctonia root rot and stalk rot	Rhizoctonia solani, Rhizoctonia zeae
Root rots, minor	Alternaria alternata, Cercospora sorghi, Dictochaeta fertilis, Fusarium acuminatum (teleomorph: Gibberella acuminata), F. equiseti (teleomorph: G. intricans), F. oxysporum, F. pallidoroseum, F. poae, F. roseum, G. cyanogena, (anamorph: F. sulphureum), Microdochium bolleyi, Mucor sp., Periconia circinata, Phytophthora cactorum, P. drechsleri, P. nicotianae var. parasitica, Rhizopus arrhizus
Rostratum leaf spot (Helminthosporium leaf disease, ear and stalk rot)	Setosphaeria rostrata, (anamorph: Exserohilum rostratum = Helminthosporium rostratum)
Rust, common corn	Puccinia sorghi
Rust, southern corn	Puccinia polysora
Rust, tropical corn	Physopella pallescens, P. zeae = Angiopsora zeae
Sclerotium ear rot (southern blight)	Sclerotium rolfsii Sacc. (teleomorph: Athelia rolfsii)
Seed rot-seedling blight	Bipolaris sorokiniana, B. zeicola = Helminthosporium carbonum, Diplodia maydis, Exserohilum pedicillatum, Exserohilum turcicum = Helminthosporium turcicum, Fusarium avenaceum, F. culmorum, F. moniliforme, Gibberella zeae (anamorph: F. graminearum), Macrophomina phaseolina, Penicillium spp., Phomopsis sp., Pythium spp., Rhizoctonia solani, R. zeae, Sclerotium rolfsii, Spicaria sp.

Disease	Pathogen
Selenophoma leaf spot	Selenophoma sp.
Sheath rot	Gaeumannomyces graminis
Shuck rot	Myrothecium gramineum
Silage mold	Monascus purpureus, M ruber
Smut, common	Ustilago zeae = U. maydis
Smut, false	Ustilaginoidea virens
Smut, head	Sphacelotheca reiliana = Sporisorium holcisorghi
Southern corn leaf blight and stalk rot	Cochliobolus heterostrophus (anamorph: Bipolaris maydis = Helminthosporium maydis)
Southern leaf spot	Stenocarpella macrospora = Diplodia macrospora
Stalk rots, minor	Cercospora sorghi, Fusarium episphaeria, F. merismoides, F. oxysporum Schlechtend, F. poae, F. roseum, F. solani (teleomorph: Nectria haematococca), F. tricinctum, Mariannaea elegans, Mucor sp., Rhopographus zeae, Spicaria sp.
Storage rots	Aspergillus spp., Penicillium spp. and other fungi
Tar spot	Phyllachora maydis
Trichoderma ear rot and root rot	Trichoderma viride = T. lignorum teleomorph: Hypocrea sp.
White ear rot, root and stalk rot	Stenocarpella maydis = Diplodia zeae
Yellow leaf blight	Ascochyta ischaemi, Phyllosticta maydis (teleomorph: Mycosphaerella zeae-maydis)
Zonate leaf spot	Gloeocercospora sorghi

The following are especially preferred

- Plasmodiophoromycota such as Plasmodiophora brassicae (clubroot of crucifers), Spongospora subterranea (powdery scab of potato tubers), Polymyxa graminis (root disease of cereals and grasses),

- Oomycota such as Bremia lactucae (downy mildew of lettuce), Peronospora (downy mildew) in snapdragon (P. antirrhini), onion (P. destructor), spinach (P. effusa), soybean (P. manchurica), tobacco ("blue mold"; P. tabacina), alfalfa and clover (P. trifolium), Pseudoperonospora humuli (downy mildew of hops), Plasmopara (downy mildew in grapevines) (P.

viticola) and sunflower (P. halstedii), Sclerophtohra macrospora (downy mildew in cereals and grasses), Pythium (seed rot, seedling damping-off, and root rot of all types of plants, for example damping-off of Beta beet caused by P. debaryanum), Phytophthora infestans (blight in potato, brown rot in tomato and the like), Albugo spec. (white rust on cruciferous plants).

- Ascomycota such as Microdochium nivale (snow mold of rye and wheat), Fusarium graminearum, Fusarium culmorum (partial ear sterility mainly in wheat), Fusarium oxysporum (Fusarium wilt of tomato), Blumeria graminis (powdery mildew of barley (f.sp. hordei) and wheat (f.sp. tritici)), Erysiphe pisi (powdery mildew of pea), Nectria galligena (Nectria canker of fruit trees), Uncinula necator (powdery mildew of grapevine), Pseudopeziza tracheiphila (red fire disease of grapevine), Claviceps purpurea (ergot on, for example, rye and grasses), Gaeumannomyces graminis (take-all on wheat, rye and other grasses), Magnaporthe grisea (rice blast disease), Pyrenophora graminea (leaf stripe of barley), Pyrenophora teres (net blotch of barley), Pyrenophora tritici-repentis (leaf blight of wheat), Venturia inaequalis (apple scab), Sclerotinia sclerotium (stalk break, stem rot), Pseudopeziza medicaginis (leaf spot of alfalfa, white and red clover).

- Basidiomycetes such as Typhula incarnata (typhula blight on barley, rye, wheat), Ustilago maydis (blister smut on maize), Ustilago nuda (loose smut on barley), Ustilago tritici (loose smut on wheat, spelt), Ustilago avenae (loose smut on oats), Rhizoctonia solani (rhizoctonia root rot of potato), Sphacelotheca spp. (head smut of sorghum), Melampsora lini (rust of flax), Puccinia graminis (stem rust of wheat, barley, rye, oats), Puccinia recondita (leaf rust on wheat), Puccinia dispersa (brown rust on rye), Puccinia hordei (leaf rust of barley), Puccinia coronata (crown rust of oats), Puccinia striiformis (yellow rust of wheat, barley, rye and a large number of grasses), Uromyces appendiculatus (brown rust of bean), Sclerotium rolfsii (root and stem rots of many plants).

- Deuteromycetes (Fungi imperfecti) such as Septoria nodorum (glume blotch) of wheat (Septoria tritici), Pseudocercosporella herpotrichoides (eyespot of wheat, barley, rye), Rynchosporium secalis (leaf spot on rye and barley), Alternaria solani (early blight of potato, tomato), Phoma betae (blackleg on Beta beet), Cercospora beticola (leaf spot on Beta beet), Alternaria brassicae (black spot on

oilseed rape, cabbage and other crucifers), Verticillium dahliae (verticillium wilt), Colletotrichum lindemuthianum (bean anthracnose), Phoma lingam (blackleg of cabbage and oilseed rape), Botrytis cinerea (gray mold of grapevine, strawberry, tomato, hops and the like).

Most preferred are Phytophthora infestans (potato blight, brown rot in tomato and the like), Microdochium nivale (previously Fusarium nivale; snow mold of rye and wheat), Fusarium graminearum, Fusarium culmorum (partial ear sterility of wheat), Fusarium oxysporum (Fusarium wilt of tomato), Blumeria graminis (powdery mildew of barley (f. sp. hordei) and wheat (f. sp. tritici)), Magnaporthe grisea (rice blast disease), Sclerotinia sclerotium (stalk break, stem rot), Septoria nodorum and Septoria tritici (glume blotch of wheat), Alternaria brassicae (black spot of oilseed rape, cabbage and other crucifers), Phoma lingam (blackleg of cabbage and oilseed rape).

2. Bacterial pathogens:

The pathogens and diseases associated with them, all of which are mentioned in table 3, may be mentioned by way of example, but not by limitation.

Table 3: Bacterial diseases

Disease	Pathogen
Bacterial leaf blight and stalk rot	Pseudomonas avenae subsp. avenae
Bacterial leaf spot	Xanthomonas campestris pv. holcicola
Bacterial stalk rot	Enterobacter dissolvens = Erwinia dissolvens
Bacterial stalk and top rot	Erwinia carotovora subsp. carotovora, Erwinia chrysanthemi pv. zeae
Bacterial stripe	Pseudomonas andropogonis
Chocolate spot	Pseudomonas syringae pv. coronafaciens
Goss's bacterial wilt and blight (leaf freckles and wilt)	Clavibacter michiganensis subsp. nebraskensis = Corynebacterium michiganense pv.nebraskense
Holcus spot	Pseudomonas syringae pv. syringae
Purple leaf sheath	Hemiparasitic bacteria
Seed rot-seedling blight	Bacillus subtilis

Stewart's disease (bacterial wilt)	Pantoea stewartii = Erwinia stewartii
Corn stunt (achapparramiento, maize stunt, Mesa Central or Rio Grande maize stunt)	Spiroplasma kunkelii

Very especially preferred are the following pathogenic bacteria: Corynebacterium sepedonicum (potato bacterial ring rot), Erwinia carotovora (potato bacterial soft rot), Erwinia amylovora (fire blight on pear, apple, quince), Streptomyces scabies (potato scab), Pseudomonas syringae pv. tabaci (tobacco black fire), Pseudomonas syringae pv. phaseolicola (bean grease spot), Pseudomonas syringae pv. tomato (tomato bacterial speck), Xanthomonas campestris pv. malvacearum (cotton bacterial blight) and Xanthomonas campestris pv. oryzae (bacterial leaf blight on rice and other grasses).

3. Viral pathogens:

"Viral pathogens" includes all plant viruses such as, for example, tobacco or cucumber mosaic virus, ringspot virus, necroses virus, maize dwarf mosaic virus and the like.

Pathogens and the diseases associated with them may be mentioned in table 4 by way of example, but not by limitation.

Table 4: Viral diseases

Disease	Pathogen
American wheat striate (wheat striate mosaic)	American wheat striate mosaic virus (AWSMV)
Barley stripe mosaic	Barley stripe mosaic virus (BSMV)
Barley yellow dwarf	Barley yellow dwarf virus (BYDV)
Brome mosaic	Brome mosaic virus (BMV)
Cereal chlorotic mottle	Cereal chlorotic mottle virus (CCMV)
Corn chlorotic vein banding (Braizilian maize mosaic)	Corn chlorotic vein banding virus (CCVBV)
Corn lethal necrosis	Virus complex of Maize chlorotic mottle virus (MCMV) and Maize dwarf mosaic virus (MDMV) A or B or Wheat streak mosaic virus(WSMV)
Cucumber mosaic	Cucumber mosaic virus (CMV)
Cynodon chlorotic streak	Cynodon chlorotic streak virus (CCSV)
Johnsongrass mosaic	Johnsongrass mosaic virus (JGMV)

Disease	Pathogen
Maize bushy stunt	Mycoplasma-like organism (MLO) associated
Maize chlorotic dwarf	Maize chlorotic dwarf virus (MCDV)
Maize chlorotic mottle	Maize chlorotic mottle virus (MCMV)
Maize dwarf mosaic	Maize dwarf mosaic virus (MDMV) strains A, D, E and F
Maize leaf fleck	Maize leaf fleck virus (MLFV)
Maize line	Maize line virus (MLV)
Maize mosaic (corn leaf stripe, enanismo rayado)	Maize mosaic virus (MMV)
Maize mottle and chlorotic stunt	Maize mottle and chlorotic stunt virus
Maize pellucid ringspot	Maize pellucid ringspot virus (MPRV)
Maize raya gruesa	Maize raya gruesa virus (MRGV)
maize rayado fino (fine striping disease)	Maize rayado fino virus (MRFV)
Maize red leaf and red stripe	Mollicute
Maize red stripe	Maize red stripe virus (MRSV)
Maize ring mottle	Maize ring mottle virus (MRMV)
Maize rio IV	Maize rio cuarto virus (MRCV)
Maize rough dwarf (nanismo ruvido)	Maize rough dwarf virus (MRDV) (Cereal tillering disease virus)
Maize sterile stunt	Maize sterile stunt virus (strains of barley yellow striate virus)
Maize streak	Maize streak virus (MSV)
Maize stripe (maize chlorotic stripe, maize hoja blanca)	Maize stripe virus
Maize stunting	Maize stunting virus
Maize tassel abortion	Maize tassel abortion virus (MTAV)
Maize vein enation	Maize vein enation virus (MVEV)
Maize wallaby ear	Maize wallaby ear virus (MWEV)
Maize white leaf	Maize white leaf virus
Maize white line mosaic	Maize white line mosaic virus (MWLMV)
Millet red leaf	Millet red leaf virus (MRLV)
Northern cereal mosaic	Northern cereal mosaic virus (NCMV)
Oat pseudorosette (zakuklivanie)	Oat pseudorosette virus
Oat sterile dwarf	Oat sterile dwarf virus (OSDV)
Rice black-streaked dwarf	Rice black-streaked dwarf virus (RBSDV)
Rice stripe	Rice stripe virus (RSV)

Disease	Pathogen
Sorghum mosaic	Sorghum mosaic virus (SrMV) (also: sugarcane mosaic virus (SCMV) strains H, I and M)
Sugarcane Fiji disease	Sugarcane Fiji disease virus (FDV)
Sugarcane mosaic	Sugarcane mosaic virus (SCMV) strains A, B, D, E, SC, BC, Sabi and MB (formerly MDMV-B)
Wheat spot mosaic	Wheat spot mosaic virus (WSMV)

4. Animal pests

4.1 Insect pathogens:

Insects such as, for example, beetles, caterpillars, lice or mites may be mentioned by way of example, but not by limitation. Preferred are insects of the genera Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, and the like. Especially preferred are Coleoptera and Lepidoptera insects such as, for example, the European corn borer (ECB), Diabrotica barberi ("northern corn rootworm"), Diabrotica undecimpunctata ("southern corn rootworm"), Diabrotica virgifera ("Western corn rootworm"), Agrotis ipsilon ("black cutworm"), Crymodes devastator ("glassy cutworm"), Feltia ducens ("dingy cutworm"), Agrotis gladiaria ("claybacked cutworm"), Melanotus spp., Aeolus mellillus ("wireworm"), Aeolus mancus ("wheat wireworm"), Horistonotus uhlerii ("sand wireworm"), Sphenophorus maidis ("maize billbug"), Sphenophorus zeae ("timothy billbug"), Sphenophorus parvulus ("bluegrass billbug"), Sphenophorus callosus ("southern corn billbug"), Phyllogphaga spp. ("white grubs"), Anuraphis maidiradicis ("corn root aphid"), Delia platura ("seedcorn maggot"), Colaspis brunnea ("grape colaspis"), Stenolophus lecontei ("seedcorn beetle") and Clivinia impressifrons ("lender seedcorn beetle").

Others which may be mentioned are: the cereal leaf beetle (Oulema melanopus), the frit fly (Oscinella frit), wireworms (Agrotis lineatus) and aphids (such as, for example, the oat grain aphid Rhopalosiphum padi, the blackberry aphid Sitobion avenae).

4.2 Nematodes:

Pathogens and the diseases associated with them may be mentioned by way of example, but not by way of limitation, in table 6.

Table 6: Parasitic nematodes

Disease	Pathogenic Nematode
Awl	Dolichodorus spp., D. heterocephalus
Bulb and stem nematode disease; Europe	Ditylenchus dipsaci
Burrowing	Radopholus similis
Cyst nematode disease	Heterodera avenae, H. zeae, Punctodera chalcoensis
Dagger	Xiphinema spp., X. americanum, X. mediterraneum
False root-knot	Nacobbus dorsalis
Lance, Columbia	Hoplolaimus columbus
Lance	Hoplolaimus spp., H. galeatus
Lesion	Pratylenchus spp., P. brachyurus, P. crenatus, P. hexincisus, P. neglectus, P. penetrans, P. scribneri, P. thornei, P. zeae
Needle	Longidorus spp., L. breviannulatus
Ring	Criconemella spp., C. ornata
Root-knot disease	Meloidogyne spp., M. chitwoodi, M. incognita, M. javanica
Spiral	Helicotylenchus spp.
Sting	Belonolaimus spp., B. longicaudatus
Stubby-root	Paratrichodorus spp., P. christiei, P. minor, Quinisulcius acutus, Trichodorus spp.
Stunt	Tylenchorhynchus dubius

Very especially preferred are Globodera rostochiensis and G. pallida (cyst eelworm on potato, tomato and other Solanaceae), Heterodera schachtii (beet eelworm on sugar and fodder beet, oilseed rape, cabbage and the like), Heterodera avenae (cereal cyst nematode on oat and other cereal species), Ditylenchus dipsaci (stem or bulb eelworm, stem eelworm of rye, oats, maize, clover, tobacco, beet), Anguina tritici (ear-cockle nematode, cockle disease of wheat (spelt, rye), Meloidogyne hapla (root-knot nematode of carrot, cucumber, lettuce, tomato, potato, sugar beet, alfalfa).

Examples of fungal or viral pathogens which are preferred for the individual varieties are the following:

1. Barley:

fungal, bacterial and viral pathogens: Puccinia graminis f.sp. hordei (barley stem rust), Blumeria (Erysiphe) graminis f.sp. hordei (Barley Powdery Mildew), barley yellow dwarf virus (BYDV),

Pathogenic insects / nematodes: Ostrinia nubilalis (European corn borer); Agrotis ipsilon (black cutworm); Schizaphis graminum (greenbug); Blissus leucopterus leucopterus (chinch bug); Acrosternum hilare (green stink bug); Euschistus servus (brown stink bug); Deliaplatura (seedcorn maggot); Mayetiola destructor (Hessian fly); Petrobia latens (brown wheat mite).

2. Soybean:

Fungal, bacterial or viral pathogens: Phytophthora megasperma fsp. glycinea, Macrophomina phaseolina, Rhizoctonia solani, Sclerotinia sclerotiorum, Fusarium oxysporum, Diaporthe phaseolorum var. sojae (Phomopsis sojae), Diaporthe phaseolorum var. caulivora, Sclerotium rolfsii, Cercospora kikuchii, Cercospora sojina, Peronospora manchurica, Colletotrichum dematium (Colletotrichum truncatum), Corynespora cassiicola, Septoria glycines, Phyllosticta sojicola, Alternaria alternata, Pseudomonas syringae p.v. glycinea, Xanthomonas campestris p.v. phaseoli, Microsphaera diffussa, Fusarium semitectum, Phialophora gregata, Soybean mosaic virus, Glomerella glycines, Tobacco Ring spot virus, Tobacco Streak virus, Phakopsorapachyrhizi, Pythium aphanidermatum, Pythium ultimum, Pythium debaryanum, Tomato spotted wilt virus, Heterodera glycines, Fusarium solani.

Pathogenic insects / nematodes: Pseudoplusia includens (soybean looper); Anticarsia gemmatalis (velvetbean caterpillar); Plathypena scabra (green cloverworm); Ostrinia nubilalis (European corn borer); Agrotis ipsilon (black cutworm); Spodoptera exigua (beet armyworm); Heliothis virescens (cotton budworm); Helicoverpa zea (cotton bollworm); Epilachna varivestis (Mexican bean beetle); Myzus persicae (green peach aphid); Empoasca fabae (potato leaf hopper); Acrosternum hilare (green stink bug); Melanoplus femurrubrum (redlegged grasshopper); Melanoplus differentialis (differential grasshopper); Hylemya platura (seedcorn maggot); Sericothrips variabilis (soybean thrips); Thrips tabaci (onion thrips); Te-

tranychus turkestani (strawberry spider mite); Tetranychus urticae (two-spotted spider mite).

3. Canola:

Fungal, bacterial or viral pathogens: Albugo candida, Alternaria brassicae, Leptosphaeria maculans, Rhizoctonia solani, Sclerotinia sclerotiorum, Mycosphaerella brassiccola, Pythium ultimum, Peronospora parasitica, Fusarium roseum, Alternaria alternata.

4. Alfalfa:

Fungal, bacterial or viral pathogens: Clavibacter michiganense subsp. insidiosum, Pythium ultimum, Pythium irregulare, Pythium splendens, Pythium debaryanum, Pythium aphanidermatum, Phytophthora megasperma, Peronospora trifoliorum, Phoma medicaginis var. medicaginis, Cercospora medicaginis, Pseudopeziza medicaginis, Leptotrochila medicaginis, Fusarium, Xanthomonas campestris p.v. alfalfae, Aphanomyces euteiches, Stemphylium herbarum, Stemphylium alfalfae.

5. Wheat:

Fungal, bacterial or viral pathogens: Pseudomonas syringae p.v. atrofaciens, Urocystis agropyri, Xanthomonas campestris p.v. translucens, Pseudomonas syringae p.v. syringae, Alternaria alternata, Cladosporium herbarum, Fusarium graminearum, Fusarium avenaceum, Fusarium culmorum, Ustilago tritici, Ascochyta tritici, Cephalosporium gramineum, Collotetrichum graminicola, Erysiphe graminis f.sp. tritici, Puccinia graminis f.sp. tritici, Puccinia recondita f.sp. tritici, Puccinia striiformis, Pyrenophora tritici-repentis, Septoria nodorum, Septoria tritici, Septoria avenae, Pseudocercosporella herpotrichoides, Rhizoctonia solani, Rhizoctonia cerealis, Gaeumannomyces graminis var. tritici, Pythium aphanidermatum, Pythium arrhenomanes, Pythium ultimum, Bipolaris sorokiniana, Barley Yellow Dwarf Virus, Brome Mosaic Virus, Soil-Borne Wheat Mosaic Virus, Wheat Streak Mosaic Virus, Wheat Spindle Streak Virus, American Wheat Striate Virus, Claviceps purpurea, Tilletia tritici, Tilletia laevis, Ustilago tritici, Tilletia indica, Rhizoctonia solani, Pythium arrhenomannes, Pythium gramicola, Pythium aphanidermatum, High Plains Virus, European wheat striate virus, Puccinia graminis f.sp. tritici (Wheat stem rust), Blumeria (Erysiphe) graminis f.sp. tritici (Wheat Powdery Mildew)

Pathogenic insects / nematodes: Pseudaletia unipunctata (army worm); Spodoptera frugiperda (fall armyworm); Elasmopalpus lignosellus (lesser cornstalk borer); Agrotis orthogonia (western cutworm); Elasmopalpus Zignosellus (lesser cornstalk borer); Oulema melanopus (cereal leaf beetle); Hypera punctata (clover leaf weevil); Diabrotica undecimpunctata howardi (southern corn rootworm); Russian wheat aphid; Schizaphis graminum (greenbug); Macrosiphum avenae (English grain aphid); Melanoplus femurrubrum (redlegged grasshopper); Melanoplus differentialis (differential grasshopper); Melanoplus sanguinipes (migratory grasshopper); Mayetiola destructor (Hessian fly); Sitodiplosis mosellana (wheat midge); Meromyza americana (wheat stem maggot); Hylemya coarctata (wheat bulb fly); Frankliniella fusca (tobacco thrips); Cephus cinctus (wheat stem sawfly); Aceria tulipae (wheat curl mite).

6. Sunflower:

Fungal, bacterial or viral pathogens: Plasmophora halstedii, Sclerotinia sclerotiorum, Aster Yellows, Septoria helianthi, Phomopsis helianthi, Alternaria helianthi, Alternaria zinniae, Botrytis cinerea, Phoma macdonaldii, Macrophomina phaseolina, Erysiphe cichoracearum, Rhizopus oryzae, Rhizopus arrhizus, Rhizopus stolonifer, Puccinia helianthi, Verticillium dahliae, Erwinia carotovorum p.v. Carotovora, Cephalosporium acremonium, Phytophthora cryptogea, Albugo tragopogonis.

Pathogenic insects / nematodes: Suleima helianthana (sunflower bud moth); Homoeosoma electellum (sunflower moth); zygogramma exclamationis (sunflower beetle); Bothyrus gibbosus (carrot beetle); Neolasioptera murtfeldtiana (sunflower seed midge).

7. Maize:

Fungal, bacterial or viral pathogens: Fusarium moniliforme var. subglutinans, Erwinia stewartii, Fusarium moniliforme, Gibberella zeae (Fusarium graminearum), Stenocarpella maydi (Diplodia maydis), Pythium irregulare, Pythium debaryanum, Pythium graminicola, Pythium splendens, Pythium ultimum, Pythium aphanidermatum, Aspergillus flavus, Bipolaris maydis 0, T (Cochliobolus heterostrophus), Helminthosporium carbonum I, II & III (Cochliobolus carbonum), Exserohilum turcicum I, II & III, Helminthosporium pedicellatum, Physoderma maydis, Phyllosticta maydis, Kabatiella maydis, Cercospora sorghi, Ustilago maydis, Puccinia sorghi, Puccinia polysora, Macro-

phomina phaseolina, Penicillium oxalicum, Nigrospora oryzae, Cladosporium herbarum, Curvularia lunata, Curvularia inaequalis, Curvularia pallescens, Clavibacter michiganense subsp. nebraskense, Trichoderma viride, Maize Dwarf Mosaic Virus A & B, Wheat Streak Mosaic Virus, Maize Chlorotic Dwarf Virus, Claviceps sorghi, Pseudonomas avenae, Erwinia chrysanthemi p.v. Zea, Erwinia corotovora, Cornstunt spiroplasma, Diplodia macrospora, Sclerophthora macrospora, Peronosclerospora sorghi, Peronosclerospora philippinesis, Peronosclerospora maydis, Peronosclerospora sacchari, Spacelotheca reiliana, Physopella zeae, Cephalosporium maydis, Caphalosporium acremonium, Maize Chlorotic Mottle Virus, High Plains Virus, Maize Mosaic Virus, Maize Rayado Fino Virus, Maize Streak Virus (MSV), Maize Stripe Virus, Maize Rough Dwarf Virus.

Pathogenic insects / nematodes: Ostrinia nubilalis (European corn borer); Agrotis ipsilon (black cutworm); Helicoverpa zea (corn earworm); Spodoptera frugiperda (fall armyworm); Diatraea grandiosella (southwestern corn borer); Elasmopalpus lignosellus (lesser cornstalk borer); Diatraea saccharalis (surgarcane borer); Diabrotica virgifera (western corn rootworm); Diabrotica longicornis barberi (northern corn rootworm); Diabrotica undecimpunctata howardi (southern corn rootworm); Melanotus spp. (wireworms); Cyclocephala borealis (northern masked chafer; white grub); Cyclocephala immaculata (southern masked chafer; white grub); Popillia japonica (Japanese beetle); Chaetocnema pulicaria (corn flea beetle); Sphenophorus maidis (maize billbug); Rhopalosiphum maidis (corn leaf aphid); Anuraphis maidiradicis (corn root aphid); Blissus leucopterus leucopterus (chinch bug); Melanoplus femurrubrum (redlegged grasshopper); Melanoplus sanguinipes (migratory grasshopper); Hylemva platura (seedcorn maggot); Agromyza parvicornis (corn blot leafminer); Anaphothrips obscrurus (grass thrips); Solenopsis milesta (thief ant); Tetranychus urticae (twospotted spider mite).

8. Sorghum:

Fungal, bacterial or viral pathogens: Exserohilum turcicum, Colletotrichum graminicola (Glomerella graminicola), Cercospora sorghi, Gloeocercospora sorghi, Ascochyta sorghina, Pseudomonas syringae p.v. syringae, Xanthomonas campestris p.v. holcicola, Pseudomonas andropogonis, Puccinia purpurea, Macrophomina phaseolina, Perconia circinata, Fusarium moniliforme, Alternaria alternata, Bipolaris sorghicola, Helminthosporium sorghicola, Curvularia lunata, Phoma insidiosa, Pseudomonas avenae (Pseudomonas alboprecipitans), Ramulispora

sorghi, Ramulispora sorghicola, Phyllachara sacchari, Sporisorium reilianum (Sphacelotheca reiliana), Sphacelotheca cruenta, Sporisorium sorghi, Sugarcane mosaic H, Maize Dwarf Mosaic Virus A & B, Claviceps sorghi, Rhizoctonia solani, Acremonium strictum, Sclerophthona macrospora, Peronosclerospora sorghi, Peronosclerospora philippinensis, Sclerospora graminicola, Fusarium graminearum, Fusarium oxysporum, Pythium arrhenomanes, Pythium graminicola.

Pathogenic insects / nematodes: Chilo partellus (sorghum borer); Spodoptera frugiperda (fall armyworm); Helicoverpa zea (corn earworm); Elasmopalpus lignosellus (lesser cornstalk borer); Feltia subterranea (granulate cutworm); Phyllophaga crinita (white grub); Eleodes, Conoderus and Aeolus spp. (wireworm); Oulema melanopus (cereal leaf beetle); Chaetocnema pulicaria (corn flea beetle); Sphenophorus maidis (maize billbug); Rhopalosiphum maidis (corn leaf aphid); Siphaflava (yellow sugarcane aphid); Blissus leucopterus leucopterus (chinch bug); Contarinia sorghicola (sorghum midge); Tetranychus cinnabarinus (carmine spider mite); Tetranychus urticae (two-spotted spider mite).

9. Cotton:

Pathogenic insects / nematodes: Heliothis virescens (cotton budworm); Helicoverpa zea (cotton bollworm); Spodoptera exigua (beet armyworm); Pectinophora gossypiella (pink bollworm); Anthonomus grandis grandis (boll weevil); Aphis gossypii (cotton aphid); Pseudatomoscelis seriatus (cotton fleahopper); Trialeurodes abutilonea (bandedwinged whitefly); Lygus lineolaris (tarnished plant bug); Melanoplus femurrubrum (redlegged grasshopper); Melanoplus differentialis (differential grasshopper); Thrips tabaci (onion thrips); Franklinkiella fusca (tobacco thrips); Tetranychus cinnabarinus (carmine spider mite); Tetranychus urticae (twospotted spider mite).

10. Rice:

Pathogenic insects / nematodes: Diatraea saccharalis (sugarcane borer); Spodoptera frugiperda (fall armyworm); Helicoverpa zea (corn earworm); Colaspis brunnea (grape colaspis); Lissorhoptrus oryzophilus (rice water weevil); Sitophilus oryzae (rice weevil); Nephotettix nigropictus (rice leafhopper); Blissus leucopterus leucopterus (chinch bug); Acrosternum hilare (green stink bug).

11. Oilseed rape:

Pathogenic insects / nematodes: Brevicoryne brassicae (cabbage aphid); Phyilotreta cruciferae (Flea beetle); Mamestra conjugrata (Bertha armyworm); Plutella xylostella (Diamondback moth); Delia ssp. (Root maggots).

For the purposes of the invention, "NADPH oxidase" means all those enzymes whose essential characteristic is that they are capable, by means of a single electron transfer, of converting molecular oxygen (O_2) into superoxide (O_2^-). Preferred are those enzymes which are described by the EC class E.C.1.23.45.3. In this context, the NADPH oxidases can consist of one or more polypeptides which may be identical or different.

Preferably, the NADPH oxidase is a flavocytochrome protein and comprises, as prosthetic groups, a cytochrome b and/or an FAD unit. The NADPH oxidase may consist of an $\alpha 1\beta 1$ heterodimer, the β subunit being the functional subunit of the flavocytochrome, which may comprise, as glycoprotein, the electron transport components (a hydrophilic, cytosolic, C-terminal domain, comprising NADPH and FAD, and 4 to 6 N-terminal, putative transmembrane α-helices, comprising two histidine-complexed prosthetic heme groups). The α-subunit may comprise a C-terminal, prolin-rich sequence which is capable of binding potential cytosolic, activating factors of the NADPH oxidase. Activation may take place by binding the cytosolic phox proteins (for example p47-phox, p67-phox, p40-phox) and p21rac, a GTP-binding protein.

The skilled worker is familiar with a large number of NADPH oxidases from plant organisms (Torres MA et al. (1998) Plant J 14: 365-370, inter alia). Sequences which may be mentioned by way of example, but not by limitation, are those with the following GenBank Acc. Nos.: AJ251717 (Hordeum vulgare), AP003560 (Oryza sativa var. japonica), AJ320505 (Nicotiana tabacum), AB050660 (Solanum tuberosum), AF088276 (Lycopersicon esculentum), AB008111 (Arabidopsis thaliana; Atrboh F), AF055357 (Arabidopsis thaliana; RbohD), AJ309006 (Nicotiana tabacum; rboh), AP003271 (Oryza sativa cv. japonica), AF055355 (Arabidopsis thaliana; RbohC), AF055353 (Arabidopsis thaliana; RbohA). Especially preferred are the NADPH oxidases which comprise a sequence as shown in SEQ ID: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22.

The sequences from other plants which are homologous to the NADPH oxidase sequences disclosed within the present invention can be found readily for example by database search or by screening genetic libraries using the NADPH oxidase sequences as search se-

quence or probe. Examples which may be mentioned are sequences with the following GenBank Acc. Nos.: CAC51517.1, AJ251717, T03973, BAB68079.1, AP003560, T02024, CAC87256.1, AJ320505, BAB70750.1, AB050660, AF088276_1, NP_564821.1, NM_105079, T00265 AC007764_16, NP_192862.1, NM_117194, AF147783_1, AAM28891.1, AF506374, CAC84140.1, AJ309006, T51804, NP_199602.1, NM_124165, BAB89740.1, AP003271, AAC39477.1, AF055355, NP_199919.1, NM_124485, AAC39475.1, AF055353, NP_196356.1, NM_120821, NP_194239.1, NM_118641, BAB08369.1, AB015475, AAC39478.1, AF055356, AC069143_9, NP_173357.1, NM_101781, NP_172383.1, NM_100780, AAB70398.1, AC000106, AAC39476.1, AF055354, BAB70751.1, AB050661, BAB63664.1, AP003275, AAD24966.1, AF109150.

The polypeptide sequence of the NADPH oxidase especially preferably comprises at least one sequence motif selected from the group of sequence motifs consisting of

i) AL(K/R)GL(K/R)
ii) DK(N/D)XDG(R/K)(I/L/V)(T/N)E
iii) LSASAN
iv) IMEELDP
v) K(F/L)NMA(I/L)(I/V)LXPVCRN
vi) (E/Q)WHPFSIT
vii) S(A/S)PXDD(Q/Y)(L/I)S(I/V)H(V/I/L)R
viii) DGPYG(S/A)PAGDY
ix) L(I/V)GLGIGATP
x) FYWVTREQGSF
xi) GVFYCG

The peptide sequence very especially preferably comprises at least 2 or 3, very especially preferably at least 4 or 5, most preferably all of the sequence motifs selected from the group of the sequence motifs i), ii), iii), iv), v), vi), vii), viii), ix) x) and xi). (Letters in brackets mean alternative amino acids which are possible at this position, for example (V/I) means that valine or isoleucine are possible at this position).

NADPH oxidase may also mean any other unit of an NADPH oxidase enzyme complex which is essential for activity of the NADPH oxidase.

"Protein quantity" means the amount of a NADPH oxidase polypeptide in an organism, a tissue, a cell or a cell compartment. "Reduction" of the protein quantity means the quantitative reduction of the amount of an NADPH oxidase in an organism, a tissue, a cell or a cell compartment - for example by one of the methods described hereinbelow - in comparison with the wild-type of the

same genus and species to which this method has not been applied, under otherwise identical conditions (such as, for example, culture conditions, age of the plants and the like). In this context, the reduction amounts to at least 10%, preferably at least 10% or at least 20%, especially preferably by at least 40% or 60%, very especially preferably by at least 70% or 80%, most preferably by at least 90% or 95%.

"Activity" means the ability of an NADPH oxidase of converting molecular oxygen (O_2) into superoxide (O_{2-}). "Reduction" of the activity means the reduction of the total activity of an NADPH oxidase protein in an organism, a tissue, a cell or a cell compartment - for example by one of the methods described hereinbelow - in comparison with the wild type of the same genus and species, to which this method has not been applied, under otherwise identical conditions (such as, for example, culture conditions, age of the plants and the like). In this context, the reduction amounts to at least 10%, preferably at least 10% or at least 20%, especially preferably to at least 40% or 60%, very especially preferably to at least 70% or 80%, most preferably to at least 90% or 95%.

"Function" preferably means the substrate binding capacity of an NADPH oxidase in an organism, a tissue, a cell or a cell compartment. Suitable substrates are low-molecular-weight compounds such as NADPH or FAD, but also the protein interaction partners of an NADPH oxidase.

"Reduction" of the function means, for example, the quantitative reduction of the binding capacity or binding strength of an NADPH oxidase for at least one substrate in an organism, a tissue, a cell or a cell compartment - for example by one of the methods described hereinbelow - in comparison with the wild-type of the same genus and species to which this method has not been applied, under otherwise identical conditions (such as, for example, culture conditions, age of the plants and the like). "Reduction" is also understood as meaning the change in substrate specificity as expressed, for example, by the kcat/Km value. In this context, the reduction amounts to at least 10%, preferably at least 10% or at least 20%, especially preferably to at least 40% or 60%, very especially preferably to at least 70% or 80%, most preferably to at least 90% or 95%. Binding partners for NADPH oxidase can be identified for example by the yeast-2-hybrid system in the manner with which the skilled worker is familiar.

Methods for determining the protein quantity, the activity of NADPH oxidases or the substrate binding capacity are known to the skilled worker. For example, it is possible to measure the NADPH-dependent O_2^- or H_2O_2 production which can be inhibited by DPI (for example via Nitro Blue Tetrazolium [NBT] or cytochrome c reduction). The protein quantity can be determined for example immunologically, using suitable antibodies. Suitable methods are described (Yu L et al. (1999) Blood 94(7):2497-504; Doke N (1983a) Physiol Plant Pathol 23:345-357; Levine A et al. (1994) Cell 79:583-593; Tenhaken R et al. (1995) Proc Nat Acad Sci USA 92: 4158-4163; Sagi M & Fluhr R. (2001) Plant Physiol 126(3):1281-90; Hückelhoven R & Kogel KH (1998) Mol Plant Microbe Interact 11:292-300; and references cited in the above papers).

"Functional equivalents" of an NADPH oxidase protein preferably means those sequences which are derived from an NADPH oxidase comprising a polypeptide sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22 or which are homologous with the former and which have the same essential characteristics.

In this context, the efficiency of the pathogen resistance may deviate both upwards and downwards in comparison with a value obtained when reducing one of the NADPH oxidases comprising a polypeptide sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22. Preferred functional equivalents are those where the efficiency of the pathogen resistance - measured for example with the aid of the penetration efficiency of a pathogen (development of haustora) - differs by not more than 50%, preferably 25%, especially preferably 10%, from a comparative value obtained by reducing an NADPH oxidase comprising a polypeptide sequence as described in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22. Especially preferred are those sequences whose reduction has the result that the efficiency of the pathogen resistance quantitatively exceeds a comparative value obtained by reducing one of the NAPDH oxidases comprising a polypeptide sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22 by more than 50%, preferably 100%, especially preferably 500%, very especially preferably 1000%.

The comparison is preferably carried out under analogous conditions. "Analogous conditions" means that all framework conditions such as, for example, culture or growth conditions, assay conditions (such as buffer, temperature, substrates, pathogen concentration and the like) between the experiments to be compared are kept identical and that the set-ups differ only by the sequence of the NAPDH oxidases to be compared, their organism of origin and, if appropriate, the pathogen. When selecting the

pathogen for the comparison, the pathogen to be selected for the comparison is that which is most similar to the corresponding other pathogen, taking into consideration the species specificity.

In particular, "functional equivalents" means natural or artificial mutations of the NADPH oxidases comprising a polypeptide sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22 and homologous polypeptides from other plants which continue to have essentially identical characteristics. Homologous polypeptides from the above-described preferred plants are preferred. The sequences from other plants (for example Arabidopsis thaliana) which are homologous to the NAPDH oxidase sequences disclosed within the scope of the present invention can be found readily for example by database search or screening genetic libraries, using the NADPH oxidase sequences as search sequence or probe. Such sequences are detailed above by way of example together with their GenBank Acc No.

Mutations comprise substitutions, additions, deletions, inversions or insertions of one or more amino acid residues. Thus, the present invention also comprises for example those polypeptides which are obtained by modification of a polypeptide comprising a polypeptide sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22.

Homology between two nucleic acid sequences is understood as meaning the identity of the two nucleic acid sequences over in each case the entire sequence length which is calculated by comparison with the aid of the program algorithm GAP (Wisconsin Package Version 10.0, University of Wisconsin, Genetics Computer Group (GCG), Madison, USA), setting the following parameters:

Gap weight: 50	Length weight: 3
Average match: 10	Average mismatch: 0

For example a sequence which has at least 80% homology with sequence SEQ ID NO: 1 at the nucleic acid level is understood as meaning a sequence which, upon comparison with the sequence SEQ ID NO: 1 by the above program algorithm with the above parameter set, has at least 80% homology.

Homology between two polypeptides is understood as meaning the identity of the two nucleic acid sequences over in each case the entire sequence length which is calculated by comparison with the aid of the program algorithm GAP (Wisconsin Package Version 10.0,

University of Wisconsin, Genetics Computer Group (GCG), Madison, USA; Altschul et al. (1997) Nucleic Acids Res. 25:3389 et seq.), setting the following parameters:

Gap weight: 8	Length weight: 2
Average match: 2,912	Average mismatch: -2,003

For example a sequence which has at least 80% homology with sequence SEQ ID NO: 2 at the protein level is understood as meaning a sequence which, upon comparison with the sequence SEQ ID NO: 2 by the above program algorithm with the above parameter set, has at least 80% homology.

Functional equivalents derived from an NADPH oxidase comprising a polypeptide sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22 by substitution, insertion or deletion have at least 50%, preferably at least 70%, by preference at least 90%, especially preferably at least 95%, very especially preferably at least 98% homology with a polypeptide comprising a polypeptide sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22 and are distinguished by identical essential characteristics as the former.

Functional equivalents derived from an NAPDH oxidase nucleic acid sequence comprising a sequence as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21 by substitution, insertion or deletion, have at least 50%, preferably at least 70%, by preference at least 90%, especially preferably at least 95%, very especially preferably at least 98% homology with one of the polypeptides according to the invention as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21 and encode polypeptides with the same essential characteristics as a polypeptide comprising a sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22.

Also, screening cDNA libraries or genomic libraries of other organisms, preferably of the plant species which are mentioned further below as being suitable hosts for the transformation, using the nucleic acid sequences described under SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21 or parts of these as probe, is a method known to the skilled worker for identifying homologs in other species. In this context, the probes derived from the nucleic acid sequences as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21 have a length of at least 20 bp, preferably at least 50 bp, especially preferably at least 100 bp, very especially preferably at least 200 bp, most preferably at least 400 bp. A DNA strand which is complementary to the sequences de-

scribed under SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21 may also be employed for screening the libraries.

Functional equivalents comprises DNA sequences which hybridize under standard conditions with the NAPDH oxidase nucleic acid sequences described under SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21, the nucleic acid sequence complementary thereto or parts of the above and which, as complete sequences, encode proteins which have the same essential characteristics as a polypeptide comprising a sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 or 22.

"Standard hybridization conditions" is to be understood in the broad sense and means stringent or else less stringent hybridization conditions. Such hybridization conditions are described, inter alia, by Sambrook J, Fritsch EF, Maniatis T et al., in Molecular Cloning (A Laboratory Manual), 2nd Edition, Cold Spring Harbor Laboratory Press, 1989, pages 9.31-9.57 or in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6.

For example, the conditions during the wash step can be selected from the range of conditions delimited by low-stringency conditions (approximately 2X SSC at 50°C) and high-stringency conditions (approximately 0.2X SSC at 50°C, preferably at 65°C) (20X SSC: 0.3M sodium citrate, 3M NaCl, pH 7.0). In addition, the temperature during the wash step can be raised from low-stringency conditions at room temperature, approximately 22°C, to higher-stringency conditions at approximately 65°C. Both of the parameters, salt concentration and temperature, can be varied simultaneously, or else one of the two parameters can be kept constant while only the other is varied. Denaturants, for example formamide or SDS, may also be employed during the hybridization. In the presence of 50% formamide, hybridization is preferably effected at 42°C. Some examples of conditions for hybridization and wash step are shown hereinbelow:

(1) Hybridization conditions can be selected, for example, from the following conditions:

a) 4X SSC at 65°C (with - optionally - 100 µg/ml denatured fragmented fish sperm DNA)

b) 6X SSC at 45°C (with - optionally - 100 µg/ml denatured fragmented fish sperm DNA),

c) 6X SSC, 0.5% SDS, 50% formamide at 42°C (with - optionally - 100 µg/ml denatured fragmented fish sperm DNA)

d) 4X SSC, 50% formamide at 42°C (with - optionally - 100 µg/ml denatured fragmented fish sperm DNA)
e) 2X or 4X SSC at 50°C (low-stringency condition),
f) 30 to 40% formamide, 2X or 4X SSC at 42°C (low-stringency condition).

(2) Wash steps can be selected, for example, from the following conditions:

a) 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS at 50°C.
b) 0.1X SSC at 65°C.
c) 0.1X SSC, 0.5% SDS at 68°C.
d) 0.1X SSC, 0.5% SDS, 50% formamide at 42°C.
e) 0.2X SSC, 0.1% SDS at 42°C.
f) 2X SSC at 65°C (low-stringency condition).

The reduction of the expression of an NADPH oxidase protein, the NADPH oxidase activity or the NADPH oxidase function can be performed in many ways.

"Reduction" or "to reduce" in connection with an NADPH oxidase, an NADPH oxidase activity or NADPH oxidase function is to be interpreted in the broad sense and comprises the partial or essentially complete prevention or blocking (due to a variety of cell-biological mechanisms) of the functionality of an NAPDH oxidase in a plant or a part, tissue, organ, cells or seed derived therefrom. A reduction for the purposes of the invention also comprises a quantitative reduction of an NADPH oxidase down to an essentially complete absence of the NAPDH oxidase (i.e. lack of detectability of NADPH oxidase activity or NADPH oxidase function, or lack of immunological detectability of the NADPH oxidase protein). In this context, one or more essential units of the NADPH oxidase can be reduced. In this context, the expression of a certain NADPH oxidase or the NADPH oxidase activity or NADPH oxidase function in a cell or an organism is reduced by preferably more than 50%, especially preferably more than 80%, very especially preferably more than 90%.

A variety of strategies for reducing the expression of an NADPH oxidase protein, the NADPH oxidase activity or NADPH oxidase function are comprised in accordance with the invention. Strategies which may be mentioned by way of example, but not by limitation, are:

a) Introducing a double-stranded NADPH oxidase RNA nucleic acid sequence (NAox-dsRNA) or (an) expression cassette(s) ensuring its expression;

b) Introducing an NADPH oxidase antisense nucleic acid sequence or an expression cassette ensuring its expression. Comprised are those methods in which the antisense nucleic acid sequence is directed against an NADPH oxidase gene (that is to say, genomic DNA sequences) or an NADPH oxidase gene transcript (that is to say, RNA sequences). Also comprised are α-anomeric nucleic acid sequences.

c) Introducing an NADPH oxidase antisense nucleic acid sequence in combination with a ribozyme or an expression cassette ensuring its expression

d) Introducing NADPH oxidase sense nucleic acid sequences for inducing a cosuppression or an expression cassette ensuring their expression

e) Introducing DNA- or protein-binding factors against NADPH oxidase genes, RNAs or proteins or an expression cassette ensuring their expression

f) Introducing viral nucleic acid sequences and expression constructs bringing about the degradation of NADPH oxidase RNA, or an expression cassette ensuring their expression

g) Introducing constructs for inducing a homologous recombination at endogenous NADPH oxidase genes, for example for the generation of knock-out mutants.

h) Introducing mutations into endogenous NADPH oxidase genes for generating a loss of function (for example generation of stop codons, reading frame shifts and the like)

Here, each and every one of these methods can bring about a reduction of the NADPH oxidase expression, NADPH oxidase activity or NADPH oxidase function in the sense of the invention. A combined use is also feasible. Further methods are known to the skilled worker and can comprise hindering or preventing the processing of the NADPH oxidase protein, the transport of the NADPH oxidase protein or its mRNA, inhibition of the attachment of ribosomes, inhibition of RNA splicing, induction of an NADPH oxidase RNA degrading enzyme and/or inhibition of the translational elongation or termination.

The individual methods which are preferred shall be described briefly hereinbelow:

a) Introducing a double-stranded NADPH oxidase RNA nucleic acid sequence (NAox-dsRNA)

The method of regulating genes by means of double-stranded RNA (double-stranded RNA interference; dsRNAi) has been described many times in animal and plant organisms (for example Matzke MA et al. (2000) Plant Mol Biol 43:401-415; Fire A. et al (1998) Nature 391:806-811; WO 99/32619; WO 99/53050; WO 00/68374; WO 00/44914; WO 00/44895; WO 00/49035; WO 00/63364). The processes and methods described in the abovementioned references are expressly referred to. Efficient gene suppression can also be shown in the case of transient expression or after transient expression, for example as the result of a biolistic transformation (Schweizer P et al. (2000) Plant J 2000 24:895-903). dsRNAi methods are based on the phenomenon that the simultaneous introduction of complementary strand and counterstrand of a gene transcript brings about a highly-efficient suppression of the expression of the gene in question. The phenotype which results is very similar to a corresponding knock-out mutant (Waterhouse PM et al. (1998) Proc Natl Acad Sci USA 95:13959-64).

The dsRNAi method has proved to be particularly efficient and advantageous when reducing the NADPH oxidase expression. As described in WO 99/32619, inter alia, dsRNAi approaches are markedly superior to traditional antisense approaches.

A further aspect of the invention therefore relates to double-stranded RNA molecules (dsRNA molecules) which, when introduced into a plant (or a cell, tissue, organ or seed derived therefrom), bring about the reduction of an NADPH oxidase.

The double-stranded RNA molecule for reducing the expression of an NADPH oxidase protein comprises

a) a sense RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least part of an NADPH oxidase nucleic acid sequence, and

b) an antisense RNA strand which is essentially - preferably completely - complementary to the RNA sense strand of a).

In a furthermore preferred embodiment, the double-stranded RNA molecule for reducing the expression of an NADPH oxidase protein comprises

a) a sense RNA strand comprising at least one ribonucleotide sequence which is essentially identical to at least part of the sense RNA transcript of a nucleic acid sequence encoding an NADPH oxidase protein, and

b) an antisense RNA strand which is essentially - preferably completely - complementary to the RNA sense strand of a).

With regard to the double-stranded RNA molecules, NADPH oxidase nucleic acid sequence preferably means a sequence comprising a sequence as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21.

"Essentially identical" means that the dsRNA sequence may also have insertions, deletions and individual point mutations in comparison with the NADPH oxidase target sequence or a functional equivalent target sequence while still bringing about an efficient reduction of the expression. Preferably, the homology as defined above between the sense strand of an inhibitory dsRNA and at least part of the sense RNA transcript of a nucleic acid sequence encoding an NAPDH oxidase protein or functional equivalent thereof (or between the antisense strand of the complementary strand of a nucleic acid sequence encoding an NAPDH oxidase protein or a functional equivalent thereof) amounts to at least 75%, preferably at least 80%, very especially preferably at least 90% most preferably 100%.

The length of the part-segment amounts to at least 10 bases, preferably at least 25 bases, especially preferably at least 50 bases, very especially preferably at least 100 bases, most preferably at least 200 bases or at least 300 bases.

Alternatively, an "essentially identical" dsRNA may also be defined as a nucleic acid sequence which is capable of hybridizing with a part of a storage protein gene transcript (for example in 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA at 50°C or 70°C for 12 to 16 h).

"Essentially complementary" means that the antisense RNA strand may also have insertions, deletions and individual point mutations in comparison with the complement of the sense RNA strand. Preferably, the homology between the antisense RNA strand and the complement of the sense RNA strand amounts to at least 80%, pre-

ferably at least 90%, very especially preferably at least 95%, most preferably 100%.

"Part of the sense RNA transcript" of a nucleic acid sequence encoding an NADPH oxidase protein or a functional equivalent thereof means fragments of an RNA or mRNA transcribed from a nucleic acid sequence encoding an NADPH oxidase protein or a functional equivalent thereof, preferably an NADPH oxidase gene. Here, the fragments preferably have a sequence length of at least 20 bases, preferably at least 50 bases, especially preferably at least 100 bases, very especially preferably at least 200 bases, most preferably at least 500 bases. Also comprised is the complete transcribed RNA or mRNA.

Also comprised is the use of the dsRNA molecules according to the invention in the methods according to the invention for generating a pathogen resistance in plants.

The dsRNA can consist of one or more strands of polymerized ribonucleotides. Furthermore, modifications both of the sugar-phosphate skeleton and of the nucleosides may be present. For example, the phosphodiester bonds of the natural RNA can be modified in such a way that they comprise at least one nitrogen or sulfur heteroatom. Bases can be modified in such a way that the activity of, for example, adenosine deaminase is limited. These and further modifications are described hereinbelow in the methods for stabilizing antisense RNA.

To achieve the same purpose it is, of course, also possible to introduce, into the cell or the organism, a plurality of individual dsRNA molecules, each of which comprises one of the above-defined ribonucleotide sequence segments.

The dsRNA can be produced enzymatically or, fully or in parts, by chemical synthesis.

The double-stranded dsRNA structure can be formed starting from two complementary, separate RNA strands or - preferably - starting from a single, autocomplementary RNA strand.

In the case of a single, autocomplementary strand, sense and antisense sequence can be linked by a linking sequence (linker) and form for example a hairpin structure. Preferably, the linking sequence may be an intron, which is spliced out after the dsRNA has been synthesized.

The nucleic acid sequence encoding a dsRNA may comprise further elements such as, for example transcription termination signals or polyadenylation signals.

If the two strands of the dsRNA are to be combined in a cell or plant, this may take place in various ways, for example:

a) transformation of the cell or plant with a vector which comprises both expression cassettes,

b) cotransformation of the cell or plant with two vectors, where one comprises the expression cassettes with the sense strand and the other comprises the expression cassettes with the antisense strand,

c) hybridizing two plants, each of which has been transformed with a vector, where one comprises the expression cassettes with the sense strand and the other comprises the expression cassettes with the antisense strand.

The formation of the RNA duplex can be initiated either outside the cell or within the same. As in WO 99/53050, the dsRNA may also comprise a hairpin structure by linking sense and antisense strand by a linker (for example an intron). The autocomplementary dsRNA structures are preferred since they merely require the expression of a construct and comprise the complementary strands always in an equimolar ratio.

The expression cassettes encoding the antisense or sense strand of a dsRNA or the autocomplementary strand of the dsRNA are preferably inserted into a vector and, using the methods described hereinbelow, stably (for example using selection markers) inserted into the genome of a plant in order to ensure durable expression of the dsRNA.

The dsRNA can be introduced using such an amount that at least one copy per cell is made possible. Larger amounts (for example at least 5, 10, 100, 500 or 1000 copies per cell) may, if appropriate, bring about a more efficient reduction.

As already described, 100% sequence identity between dsRNA and an NADPH oxidase gene transcript or the gene transcript of a functionally equivalent gene is not necessarily required in order to bring about an efficient reduction of the NADPH oxidase expression. Accordingly, there is the advantage that the method tolerates sequence deviations, as may be present as the result of genetic mutations, polymorphisms or evolutionary divergences. Using

the dsRNA which has been generated starting from the NADPH oxidase sequence of an organism, it is thus, for example, possible to suppress the NAPDH oxidase expression in another organism. The high degree of sequence homology between the NADPH oxidase sequences from rice, maize and barley allows the conclusion that this protein is highly conserved within plants, so that the expression of a dsRNA derived from one of the NADPH oxidase sequences comprising a sequence as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21 probably also has an advantageous effect in other plant species.

Owing to the high degree of homology between the individual NADPH oxidase proteins and their functional equivalents, it is also possible to suppress the expression of further homologous NAPDH oxidase proteins and/or their functional equivalents of the same organism or else the expression of NAPDH oxidase proteins in other related species, using a single dsRNA which has been generated starting from a specific NADPH oxidase sequence of an organism. For this purpose, the dsRNA preferably comprises sequence regions of NADPH oxidase gene transcripts which correspond to conserved regions. Said conserved regions can be deduced readily from sequence alignments.

The dsRNA can be synthesized either in vivo or in vitro. To this end, a DNA sequence encoding a dsRNA can be introduced into an expression cassette under the control of at least one genetic control element (such as, for example, promoter, enhancer, silencer, splice donor or acceptor, polyadenylation signal). Suitable advantageous constructions are described hereinbelow. A polyadenylation is not necessary, nor do elements for initiating a translation have to be present.

A dsRNA can be synthesized chemically or enzymatically. To this end, cellular RNA polymerases or bacteriophage RNA polymerases (such as, for example, T3, T7 or SP6 RNA polymerase) can be used. Such methods for the in-vitro expression of RNA are described (WO 97/32016; US 5,593,874; US 5,698,425, US 5,712,135, US 5,789,214, US 5,804,693). A dsRNA which has been synthesized in vitro, either chemically or enzymatically, can be isolated fully or in part from the reaction mixture, for example by extraction, precipitation, electrophoresis, chromatography or combination of these methods, before it is introduced into a cell, tissue or organism. The dsRNA can be introduced directly into the cell or else by applied extracellularly (for example into the interstitial space).

However, the plant is preferably transformed stably using an expression construct which brings about the expression of the dsRNA. Suitable methods are described hereinbelow.

b) Introduction of an NADPH oxidase antisense nucleic acid sequence

Methods for suppressing a particular protein by preventing the accumulation of its mRNA by antisense technology have been described many times, also in plants (Sheehy et al. (1988) Proc Natl Acad Sci USA 85: 8805-8809; US 4,801,340; Mol JN et al. (1990) FEBS Lett 268(2):427-430). The antisense nucleic acid molecule hybridized with, or binds to, the cellular mRNA and/or genomic DNA encoding the NADPH oxidase target protein to be suppressed, which suppresses the transcription and/or translation of the target protein. The hybridization can be brought about in the traditional manner via the formation of a stable duplex or - in the case of genomic DNA - by binding the antisense nucleic acid molecule with the duplex of the genomic DNA by specific interaction in the large groove of the DNA helix.

An antisense nucleic acid sequence suitable for reducing an NADPH oxidase protein can be derived using the nucleic acid sequence which encodes this protein, for example the nucleic acid sequence comprising a sequence as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21, following the Watson-Crick base pair rules. The antisense nucleic acid sequence can be complementary to all of the transcribed mRNA of said protein, may be limited to the coding region or may consist of one oligonucleotide only, which is complementary to part of the coding or noncoding sequence of the mRNA. Thus, the oligonucleotide may, for example, be complementary to the region which comprises the translation start for said protein. Antisense nucleic acid sequences can have a length of, for example, 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 nucleotides, but may also be longer and comprise at least 100, 200, 500, 1000, 2000 or 5000 nucleotides. Antisense nucleic acid sequences can be expressed recombinantly or synthetized chemically or enzymatically using methods known to the skilled worker. In the case of chemical synthesis, natural or modified nucleotides may be used. Modified nucleotides can impart an increased biochemical stability to the antisense nucleic acid sequence and may lead to an increased physical stability of the duplex formed of antisense nucleic acid sequence and sense target sequence. Nucleotides which can be used are, for example, phosphorothioate derivatives and acridine-substituted nucleotides such as 5-fluorouracil, 5-bromouracil, 5-chlorouracil, 5-iodouracil, hypoxanthin, xanthin, 4-acetylcytosine, 5-(carboxyhydroxylmethyl)uracil,

5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethyluracil, dihydrouracil, β-D-galactosylqueosine, inosine, N6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-adenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, β-D-mannosylqueosine, 5'-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methyl ester, uracil-5-oxyacetic acid, 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl)uracil and 2,6-diaminopurine.

In a further preferred embodiment, the expression of an NADPH oxidase protein can be inhibited by nucleotide sequences which are complementary to the regulatory region of an NADPH oxidase gene (for example an NADPH oxidase promoter and/or enhancer) and form triple-helical structures with the DNA double helix therein, so that the transcription of the NADPH oxidase gene is reduced. Suitable methods are described (Helene C (1991) Anticancer Drug Res 6(6):569-84; Helene C et al. (1992) Ann NY Acad Sci 660:27-36; Maher LJ (1992) Bioassays 14(12):807-815).

In a further embodiment, the antisense nucleic acid molecule can be an α-anomeric nucleic acid. Such α-anomeric nucleic acid molecules form specific double-stranded hybrids with complementary RNA in which - in contrast to the conventional β-nucleic acids - the two strands are parallel to one another (Gautier C et al. (1987) Nucleic Acids Res 15:6625-6641). The antisense nucleic acid molecule can furthermore also comprise 2'-O-methylribonucleotides (Inoue et al. (1987) Nucleic Acids Res 15:6131-6148) or chimeric RNA-DNA analogs (Inoue et al. (1987) FEBS Lett 215:327-330).

c) Introduction of an NADPH oxidase antisense nucleic acid sequence in combination with a ribozyme

The above-described antisense strategy can advantageously be coupled with a ribozyme method. Catalytic RNA molecules or ribozymes can be adapted to any target RNA and cleave the phosphodiester backbone at specific positions, whereby the target DNA is functionally deactivated (Tanner NK (1999) FEMS Microbiol Rev 23(3):257-275). The ribozyme itself is not modified thereby, but is capable of cleaving further target RNA molecules in an analogous manner, whereby it gains the properties of an enzyme. The incorporation of ribozyme sequences into antisense RNAs imparts to these antisense-RNAs this enzyme-like, RNA-cleaving property

and thus increases their efficiency in the inactivation of the target RNA. The preparation and use of suitable ribozyme antisense RNA molecules is described for example by Haseloff et al. (1988) Nature 334:585-591.

In this manner, ribozymes (for example Hammerhead ribozymes; Haselhoff and Gerlach (1988) Nature 334:585-591) can be used for catalytically cleaving the mRNA of an enzyme to be suppressed - for example NADPH oxidase - and for preventing translation. The ribozyme technology can increase the efficiency of an antisense strategy. Methods for the expression of ribozymes for reducing certain proteins are described in (EP 0 291 533, EP 0 321 201, EP 0 360 257). Ribozyme expression in plant cells is also described (Steinecke P et al. (1992) EMBO J 11(4):1525-1530; de Feyter R et al. (1996) Mol Gen Genet. 250(3):329-338). Suitable target sequences and ribozymes can be determined for example as described by "Steinecke P, Ribozymes, Methods in Cell Biology 50, Galbraith et al. eds, Academic Press, Inc. (1995), pp. 449-460", by calculating the secondary structure of ribozyme RNA and target RNA, and by their interaction (Bayley CC et al. (1992) Plant Mol Biol. 18(2):353-361; Lloyd AM and Davis RW et al. (1994) Mol Gen Genet. 242(6):653-657). For example, it is possible to construct derivatives of the Tetrahymena L-19 IVS RNA, which have complementary regions to the mRNA of the NADPH oxidase protein to be suppressed (see also US 4,987,071 and US 5,116,742). Alternatively, such ribozymes can also be identified from a library of diverse ribozymes, using a selection process (Bartel D and Szostak JW (1993) Science 261:1411-1418).

d) Introducing an NADPH oxidase sense nucleic acid sequence for inducing a cosuppression

The expression of an NADPH oxidase nucleic acid sequence in sense orientation can lead to a cosuppression of the corresponding homologous, endogenous gene. The expression of sense RNA with homology to an endogenous gene can reduce or switch off the expression of same, similarly to what has been described for antisense approaches (Jorgensen et al. (1996) Plant Mol Biol 31(5):957-973; Goring et al. (1991) Proc Natl Acad Sci USA 88:1770-1774; Smith et al. (1990) Mol Gen Genet 224:447-481; Napoli et al. (1990) Plant Cell 2:279-289; Van der Krol et al. (1990) Plant Cell 2:291-99). Here, the introduced construct can fully or only partly represent the homologous gene to be reduced. The possibility of translation is not required. The application of this technology to plants is described for example by Napoli et al. (1990) The Plant Cell 2: 279-289 and in US 5,034,323.

Preferably, cosuppression is carried out using a sequence which is essentially identical to at least a part of the nucleic acid sequence encoding an NADPH oxidase protein or a functional equivalent thereof, for example the nucleic acid sequence comprising a sequence as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19 or 21.

e) Introducing DNA- or protein-binding factors against NADPH oxidase genes, RNAs or proteins

A reduction of an NADPH oxidase gene expression is also possible using specific DNA-binding factors, for example with factors of the zinc finger transcription factor type. These factors anneal with the genomic sequence of the endogenous target gene, preferably in the regulatory regions, and bring about a repression of the endogenous gene. The use of such a method makes it possible to reduce the expression of an endogenous NADPH oxidase gene without it being necessary to recombinantly manipulate the sequence of the latter. Suitable methods for the generation of suitable factors are described (Dreier B et al. (2001) J Biol Chem 276(31):29466-78; Dreier B et al. (2000) J Mol Biol 303(4):489-502; Beerli RR et al. (2000) Proc Natl Acad Sci USA 97 (4):1495-1500; Beerli RR et al. (2000) J Biol Chem 275(42):32617-32627; Segal DJ and Barbas CF 3rd. (2000) Curr Opin Chem Biol 4(1):34-39; Kang JS and Kim JS (2000) J Biol Chem 275(12):8742-8748; Beerli RR et al. (1998) Proc Natl Acad Sci USA 95(25):14628-14633; Kim JS et al. (1997) Proc Natl Acad Sci USA 94(8):3616-3620; Klug A (1999) J Mol Biol 293(2):215-218; Tsai SY et al. (1998) Adv Drug Deliv Rev 30(1-3):23-31; Mapp AK et al. (2000) Proc Natl Acad Sci USA 97(8):3930-3935; Sharrocks AD et al. (1997) Int J Biochem Cell Biol 29(12):1371-1387; Zhang L et al. (2000) J Biol Chem 275(43):33850-33860).

These factors may be selected using any desired portion of an NADPH oxidase gene. Preferably, this segment is located within the promoter region. For gene suppression, however, it may also be located in the region of the coding exons or introns. The corresponding segments are obtainable for the skilled worker by means of database search from the genetic library or - starting from an NADPH oxidase cDNA whose gene is not present in the genetic library, by screening a genomic library for corresponding genomic clones. The methods required for this purpose are known to the skilled worker.

Furthermore, it is possible to introduce, into a cell, factors which inhibit the NADPH oxidase target protein itself. The protein-binding factors may be for example aptamers (Famulok M and

Mayer G (1999) Curr Top Microbiol Immunol 243:123-36) or antibodies or antibody fragments or single-chain antibodies. The way in which these factors are obtained is described and known to the skilled worker. For example, a cytoplasmic scFv antibody has been employed for modulating the activity of the phytochrome A protein in genetically modified tobacco plants (Owen M et al. (1992) Biotechnology (N Y) 10(7):790-794; Franken E et al. (1997) Curr Opin Biotechnol 8(4):411-416; Whitelam (1996) Trend Plant Sci 1:286-272).

Gene expression can also be suppressed by tailor-made low-molecular-weight synthetic compounds, for example of the polyamide type (Dervan PB and Bürli RW (1999) Current Opinion in Chemical Biology 3:688-693; Gottesfeld JM et al. (2000) Gene Expr 9(1-2):77-91). These oligomers consist of the units 3-(dimethylamino)propylamine, N-methyl-3-hydroxypyrrole, N-methylimidazole and N-methylpyrrole and can be adapted to any portion of double-stranded DNA in such a way that they bind sequence-specifically in the large groove and block the expression of the gene sequences which are located therein. Suitable methods are described (see, inter alia, Bremer RE et al. (2001) Bioorg Med Chem. 9(8):2093-103; Ansari AZ et al. (2001) Chem Biol. 8(6):583-92; Gottesfeld JM et al. (2001) J Mol Biol. 309(3):615-29; Wurtz NR et al. (2001) Org Lett 3(8):1201-3; Wang CC et al. (2001) Bioorg Med Chem 9(3):653-7; Urbach AR and Dervan PB (2001) Proc Natl Acad Sci USA 98(8):4343-8; Chiang SY et al. (2000) J Biol Chem. 275(32):24246-54).

f) Introducing viral nucleic acid sequences and expression constructs which bring about the degradation of NADPH oxidase RNA

NADPH oxidase expression can also be brought about efficiently by inducing the specific NADPH oxidase RNA degradation by the plant with the aid of a viral expression system (amplicon) (Angell, SM et al. (1999) Plant J. 20(3):357-362). These systems - also referred to as "VIGS" (viral induced gene silencing) - introduce nucleic acid sequences with homology to the transcripts to be suppressed into the plant, using viral vectors. Then, transcription is switched off, probably mediated by plant defense mechanisms against viruses. Suitable techniques and methods are described (Ratcliff F et al. (2001) Plant J 25(2):237-45; Fagard M and Vaucheret H (2000) Plant Mol Biol 43(2-3):285-93; Anandalakshmi R et al. (1998) Proc Natl Acad Sci USA 95(22):13079-84; Ruiz MT (1998) Plant Cell 10(6): 937-46).

g) Introducing constructs for the induction of a homologous recombination on endogenous NADPH oxidase genes, for example for the generation of knock-out mutants.

To generate a homologously recombinant organism with reduced NADPH oxidase activity, for example a nucleic acid construct is used which comprises at least a part of the endogenous NADPH oxidase gene which is modified by a deletion, addition or substitution of at least one nucleotide in such a way that the functionality is reduced or nullified completely. The modification may also affect the regulatory elements (for example the promoter) of the gene, so that the coding sequence remains unaltered, but expression (transcription and/or translation) does not take place and is reduced.

In the case of conventional homologous recombination, the modified region is flanked at its 5' and 3' end by further nucleic acid sequences which must have a sufficient length for making possible the recombination. The length is, as a rule, in the range of from several hundred bases to several kilobases (Thomas KR and Capecchi MR (1987) Cell 51:503; Strepp et al. (1998) Proc Natl Acad Sci USA 95(8):4368-4373). For the homologous recombination, the host organism - for example a plant - is transformed with the recombination construct using the methods described hereinbelow, and clones which have undergone successful recombination are selected using, for example, an antibiotic or herbicide resistance.

Homologous recombination is a relatively rare event in higher eukaryotes, especially in plants. Random integrations into the host genome predominate. A possibility of removing the randomly integrated sequences and thus of enriching cell clones with a correct homologous recombination consists in using a sequence-specific recombination system as described in US 6,110,736, by which unspecifically integrated sequences can be deleted, which facilitates the selection of events which have integrated successfully via homologous recombination. A multiplicity of sequence-specific recombination systems can be used, examples which may be mentioned being the Cre/lox system of the bacteriophage P1, the FLP/FRT system of yeast, the Gin recombinase of the phage Mu, the Pin recombinase from E. coli and the R/RS system of the plasmid pSR1. Preferred are the bacteriophage P1 Cre/lox and the yeast FLP/FRT system. The FLP/FRT and cre/lox recombinase system has already been employed in plant systems (Odell et al. (1990) Mol Gen Genet 223: 369-378)

h) Introducing mutations into endogenous NADPH oxidase genes for generating a loss of function (for example generation of stop codons, reading frame shifts and the like)

Further suitable methods for reducing the NADPH oxidase activity are the introduction of nonsense mutations into endogenous NADPH oxidase genes, for example by means of introducing RNA/DNA oligonucleotides into the plant (Zhu et al. (2000) Nat Biotechnol 18(5):555-558), and the generation of knockout mutants with the aid of, for example, T-DNA mutagenesis (Koncz et al. (1992) Plant Mol Biol 20(5):963-976), ENU (N-ethyl-N-nitrosourea) mutagenesis or homologous recombination (Hohn B and Puchta (1999) H Proc Natl Acad Sci USA 96:8321-8323). Point mutations can also be generated by means of DNA-RNA hybrids, which are also known as chimeraplasty (Cole-Strauss et al. (1999) Nucl Acids Res 27(5):1323-1330; Kmiec (1999) Gene Therapy American Scientist 87(3):240-247).

The methods of dsRNAi, cosuppression by means of sense RNA and VIGS (virus-induced gene silencing) are also referred to as post-transcriptional gene silencing (PTGS). PTGS methods, including the reduction of the NADPH oxidase function or activity with dominant-negative NADPH oxidase variants are especially advantageous since the requirements to the homology between the endogenous gene to be suppressed and the transgenically expressed sense or dsRNA nucleic acid sequence (or between the endogeous gene and its dominant-negative variant, respectively) are lower than, for example in the case of a traditional antisense approach. Suitable homology criteria are mentioned in the description of the dsRNAi method and applied generally to PTGS methods or dominant-negative approaches. Owing to the high degree of homology between the NADPH oxidase proteins from maize, rice and barley, a high degree of conservation of these protein in plants can be deduced. Thus, using the NADPH oxidase nucleic acid sequences from barley, maize or rice, it is probably also possible efficiently to suppress the expression of homologous NADPH oxidase proteins in other species, without the isolation and structural elucidation of the NADPH oxidase homologs in these species being necessarily required. This substantially reduces the labor involved. Analogously, using dominant-negative variants of an NADPH oxidase protein from rice, maize or barley, it is presumably also possible efficiently to reduce or suppress the function/activity of its homolog in other plant species.

All substances and compounds which directly or indirectly bring about a reduction of the protein quantity, RNA quantity, gene activity or protein activity of an NADPH oxidase protein, shall hereinbelow be grouped together under the term "anti-NADPH oxi-

dase" compounds. The term "anti-NADPH oxidase" compound explicitly includes the nucleic acid sequences, peptides, proteins or other factors employed in the above-described methods.

For the purposes of the invention, "introduction" comprises all those methods which are suitable for introducing an anti-NAPDH oxidase compound directly or indirectly into a plant or a cell, compartment, tissue, organ or seed thereof, or generating it therein. Direct and indirect methods are comprised. The introduction can lead to a transient presence of an anti-NADPH-oxidase compound (for example a dsRNA) or else to a stable presence.

In accordance with the different nature of the above-described approaches, the anti-NADPH-oxidase compound can exert its function directly (for example by insertion into an endogenous NADPH oxidase gene). However, the function can also be exerted indirectly after transcription into an RNA (for example in the case of antisense approaches) or after transcription and translation into a protein (for example binding factors). Both directly and indirectly acting anti-NADPH-oxidase compounds are comprised in accordance with the invention.

"Introducing" comprises for example methods such as transfection, transduction or transformation.

Thus, for example, anti-NADPH-oxidase compounds also comprise recombinant expression constructs which bring about an expression (i.e. transcription and, if appropriate, translation) of, for example, an NADPH oxidase dsRNA or an NADPH oxidase antisense RNA - preferably in a plant or a part, tissue, organ or seed thereof.

In said expression constructs, a nucleic acid molecule whose expression (transcription and, if appropriate, translation) generates an anti-NADPH-oxidase compound, is preferably in functional linkage with at least one genetic control element (for example a promoter) which ensures expression in an organism, preferably in plants. If the expression construct is to be introduced directly into the plant and the anti-NADPH-oxidase compound (for example the NADPH oxidase dsRNA) is to be generated therein *in plantae*, then plant-specific genetic control elements (for example promoters) are preferred. However, the anti-NADPH-oxidase compound can also be generated in other organisms or *in vitro* and then be introduced into the plant (as described in Example 6 and 7). Here, preferred control elements are all those prokaryotic or eukaryotic genetic control elements (for example promoters) which

permit the expression in the organism selected in each case for the production.

Functional linkage is to be understood as meaning, for example, the sequential arrangement of a promoter with the nucleic acid sequence to be expressed (for example an anti-NAox compound) and, if appropriate, further regulatory elements such as, for example, a terminator in such a way that each of the regulatory elements can fulfill its function when the nucleic acid sequence is expressed recombinantly depending on the arrangement of the nucleic acids into sense on antisense RNA. To this end, direct linkage in the chemical sense is not necessarily required. Genetic control sequences such as, for example, enhancer sequences, can also exert their function on the target sequence from positions which are further away, or indeed from other DNA molecules. Preferred arrangements are those in which the nucleic acid sequence to be expressed recombinantly is positioned behind the sequence acting as promoter, so that the two sequences are linked covalently to each other.

Here, the distance between the promoter sequence and the nucleic acid sequence to be expressed recombinantly is less than 200 base pairs, especially preferably less than 100 base pairs, very especially preferably less than 50 base pairs.

Functional linkage, and an expression cassette, can be generated by means of customary recombination and cloning techniques as are described, for example, in Maniatis T, Fritsch EF and Sambrook J (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor (NY), in Silhavy TJ, Berman ML and Enquist LW (1984) Experiments with Gene Fusions, Cold Spring Harbor Laboratory, Cold Spring Harbor (NY), in Ausubel FM et al. (1987) Current Protocols in Molecular Biology, Greene Publishing Assoc. and Wiley Interscience and in Gelvin et al. (1990) In: Plant Molecular Biology Manual. However, further sequences which, for example, act as a linker with specific cleavage sites for restriction enzymes or as a signal peptide, may also be positioned between the two sequences. The insertion of sequences may also lead to the expression of fusion proteins. Preferably, the expression cassette, consisting of a linkage of promoter and nucleic acid sequence to be expressed, can exist in a vector-integrated form and be inserted into a plant genome, for example by transformation.

However, an expression cassette also denotes those constructions in which a promoter is placed behind an endogenous NADPH oxidase gene - for example by a homologous recombination - and the

reduction according to the invention, of an NADPH oxidase protein, is brought about by expressing an antisense NADPH oxidase RNA. Analogously, an anti-NADPH-oxidase compound (for example a nucleic acid compound encoding an NADPH oxidase dsRNA or an NADPH oxidase antisense RNA) can be placed behind an endogenous promoter in such a way that the same effect occurs. Both approaches lead to expression cassettes in the sense of the invention.

The term plant-specific promoters is understood as meaning, in principle, any promoter which is capable of governing the expression of genes, in particular foreign genes, in plants or plant parts, plant cells, plant tissues, or plant cultures. Here, expression may be, for example, constitutive, inducible or development-dependent.

The following are preferred:

a) Constitutive promoters

Preferred vectors are those which make possible a constitutive expression in plants (Benfey et al.(1989) EMBO J 8:2195-2202). "Constitutive" promoter is understood as meaning those promoters which ensure expression in a large number of, preferably all, tissues over a substantial period of plant development, preferably at all stages of plant development. In particular a plant promoter or a promoter derived from a plant virus are preferably used. Particularly preferred is the promoter of the CaMV cauliflower mosaic virus 35S transcript (Franck et al. (1980) Cell 21:285-294; Odell et al. (1985) Nature 313:810-812; Shewmaker et al. (1985) Virology 140:281-288; Gardner et al. (1986) Plant Mol Biol 6:221-228) or the 19S CaMV promoter (US 5,352,605; WO 84/02913; Benfey et al. (1989) EMBO J 8:2195-2202). Another suitable constitutive promoter is the Rubisco small subunit (SSU) promoter (US 4,962,028), the leguminB promoter (GenBank Acc. No. X03677), the Agrobacterium nopaline synthase promoter, the TR dual promoter, the Agrobacterium OCS (octopine synthase) promoter, the ubiquitin promoter (Holtorf S et al. (1995) Plant Mol Biol 29:637-649), the ubiquitin 1 promoter (Christensen et al. (1992) Plant Mol Biol 18:675-689; Bruce et al. (1989) Proc Natl Acad Sci USA 86:9692-9696), the Smas promoter, the cinnamyl alcohol dehydrogenase promoter (US 5,683,439), the promoters of the ATPase subunits or the promoter of a proline-rich protein from wheat (WO 91/13991), and further promoters of genes whose constitutive expression in plants is known to the skilled worker. Especially preferred as constitutive promoter is the promoter of the nitrilase-1 (nit1)

gene from A. thaliana (GenBank Acc. No.: Y07648.2, Nucleotide 2456-4340, Hillebrand et al. (1996) Gene 170:197-200).

b) Tissue-specific promoters

Preferred are furthermore promoters with specificity for the anthers, ovaries, flower, leaves, stems, roots and seeds.

Seed-specific promoters comprise, for example, the phaseolin promoter (US 5,504,200; Bustos MM et al. (1989) Plant Cell 1(9):839-53), the 2S albumin promoter (Joseffson LG et al. (1987) J Biol Chem 262:12196-12201), the legumin promoter (Shirsat A et al. (1989) Mol Gen Genet 215(2): 326-331), the USP (unknown seed protein) promoter (Bäumlein H et al. (1991) Mol Gen Genet 225(3):459-467, the napin promoter (US 5,608,152; Stalberg K et al. (1996) L Planta 199:515-519), the sucrose binding protein promoter (WO 00/26388), the legumin B4 promoter (LeB4; Bäumlein H et al. (1991) Mol Gen Genet 225: 121-128; Bäumlein H et al. (1992) Plant J 2(2):233-239; Fiedler U et al. (1995) Biotechnology (NY) 13(10):1090f), the Arabidopsis oleosin promoter (WO 98/45461), the Brassica Bce4 promoter (WO 91/13980). Further suitable seed-specific promoters are those of the genes encoding the high-molecular-weight glutenin (HMWG), gliadin, branching enzyme, ADP glucose pyrophosphatase (AGPase) or starch synthase. Furthermore preferred promoters are those which permit seed-specific expression in monocots such as maize, barley, wheat, rye, rice and the like. The following can be employed advantageously: the promoter of the lpt2 or lpt1 gene (WO 95/15389, WO 95/23230) or the promoters described in WO 99/16890 (promoters of the hordein gene, the glutelin gene, the oryzin gene, the prolamin gene, the gliadin gene, the glutelin gene, the zein gene, the kasirin gene, or the secalin gene).

Tuber-, storage-root- or root-specific promoters comprise, for example, the class I patatin promoter (B33), the potato cathepsin D inhibitor promoter.

Leaf-specific promoters comprise the potato cytosolic FBPase promoter (WO 97/05900), the Rubisco (ribulose-1,5-bisphosphate carboxylase) SSU (small subunit) promoter or the ST-LSI promoter from potato (Stockhaus et al. (1989) EMBO J 8:2445-2451). Very especially preferred are epidermis-specific promoters such as, for example, the OXLP gene (oxalate-oxidase-like protein) promoter (Wei et al. (1998) Plant Mol Biol 36:101-112).

Flower-specific promoters comprise, for example, the phytoene synthase promoter (WO 92/16635) or the promoter of the P-rr gene (WO 98/22593).

Anther-specific promoters comprise, for example, the 5126 promoter (US 5,689,049, US 5,689,051), the glob-1 promoter and the γ-zein promoter.

c) Chemically inducible promoters

The expression cassettes can also comprise a chemically inducible promoter (review article: Gatz et al. (1997) Annu Rev Plant Physiol Plant Mol Biol 48:89-108), by which the expression of the exogenous gene in the plant at a particular point in time can be controlled. Examples which may be mentioned are the PRP1 promoter (Ward et al. (1993) Plant Mol Biol 22:361-366), a salicylic-acid-inducible promoter (WO 95/19443), a benzenesulfonamide-inducible promoter (EP 0 388 186), a tetracyclin-inducible promoter (Gatz et al. (1992) Plant J 2:397-404), an abscisic-acid-inducible promoter (EP 0 335 528) or an ethanol- or cyclohexanone-inducible promoter (WO 93/21334).

d) Stress- or pathogen-inducible promoters

Further preferred promoters are those which are induced by biotic or abiotic stress such as, for example, the pathogen-inducible promoter of the PRP1 gene (or gst1 promotor), for example from potato (WO 96/28561; Ward et al. (1993) Plant Mol Biol 22:361-366), the tomato high-temperature-inducible hsp70 or hsp80 promoter (US 5,187,267), the potato low-temperature-inducible alpha-amylase promoter (WO 96/12814) or the light-inducible PPDK promoter. Further pathogen-inducible promoters comprise the flax *Fis1* promoter (WO 96/34949), the Vst1 promoter (Schubert et al. (1997) Plant Mol Biol 34:417-426) and the tobacco EAS4 sesquiterpene cyclase promoter (US 6,100,451).

Pathogen-inducible promoters furthermore comprise the promoters of genes which are induced as a consequence of infection by pathogens, such as, for example, genes of PR proteins, SAR proteins, β-1,3-glucanase, chitinase and the like (for example Redolfi et al. (1983) Neth J Plant Pathol 89:245-254; Uknes et al. (1992) Plant Cell 4:645-656; Van Loon (1985) Plant Mol Viral 4:111-116; Marineau et al. (1987) Plant Mol Biol 9:335-342; Matton et al. (1987) Molecular Plant-Microbe Interactions 2:325-342; Somssich et al. (1986) Proc Natl Acad Sci USA 83:2427-2430; Somssich et al. (1988) Mol Gen Genetics 2:93-98; Chen et al. (1996) Plant J 10:955-966; Zhang and Sing (1994) Proc

Natl Acad Sci USA 91:2507-2511; Warner, et al. (1993) Plant J 3:191-201; Siebertz et al. (1989) Plant Cell 1:961-968(1989).

Also comprised are wounding-inducible promoters such as that of the pinII gene (EP-A 0 375 091; Ryan (1990) Ann Rev Phytopath 28:425-449; Duan et al. (1996) Nat Biotech 14:494-498), of the wun1 and wun2 gene (US 5,428,148), of the win1 and win2 gene (Stanford et al. (1989) Mol Gen Genet 215:200-208), of the systemin gene (McGurl et al. (1992) Science 225:1570-1573), of the WIP1 gene (Rohmeier et al. (1993) Plant Mol Biol 22:783-792; Eckelkamp et al. (1993) FEBS Letters 323:73-76), of the MPI gene (Corderok et al. (1994) Plant J 6(2):141-150) and the like.

A source of further pathogen-inducible promoters is the PR gene family. A series of elements in these promoters has proved to be advantageous. Thus, the region -364 to -288 in the promoter of PR-2d mediates salicylate specificity (Buchel et al. (1996) Plant Mol Biol 30, 493-504). The sequence 5'-TCATCTTCTT-3' occurs repeatedly in the promoter of the barley β-1,3-glucanase and in more than 30 further stress-induced genes. In tobacco, this region binds a nuclear protein whose abundance is increased by salicylate. The PR-1 promoters from tobacco and Arabidopsis (EP-A 0 332 104, WO 98/03536) are likewise suitable as pathogen-inducible promoters. Preferred, since especially specifically pathogen-induced, are the acidic *PR-5* (aPR5) promoters from barley (Schweizer et al. (1997) Plant Physiol 114:79-88) and wheat (Rebmann et al. (1991) Plant Mol Biol 16:329-331). aPR5 proteins accumulate in approximately 4 to 6 hours after pathogen attack and show only very little background expression (WO 99/66057). An approach for achieving an increased pathogen-induced specificity is the generation of synthetic promoters from combinations of known pathogen-responsive elements (Rushton et al. (2002) Plant Cell 14, 749-762; WO 00/01830; WO 99/66057). Further pathogen-inducible promoters from different species are known to the skilled worker (EP-A 1 165 794; EP-A 1 062 356; EP-A 1 041 148; EP-A 1 032 684).

e) Development-dependent promoters

Further suitable promoters are, for example, fruit-maturation-specific promoters such as, for example, the tomato fruit-maturation-specific promoter (WO 94/21794, EP 409 625). Development-dependent promoters comprise partly the tissue-specific promoters since individual tissues develop by nature in a development-dependent fashion.

Especially preferred are constitutive promoters and also leaf- and/or stem-specific, pathogen-inducible and epidermis-specific promoters, with pathogen-inducible and epidermis-specific promoters being most preferred.

Furthermore, further promoters may be linked functionally to the nucleic acid sequence to be expressed, which promoters make possible an expression in further plant tissues or in other organisms, such as, for example, *E. coli* bacteria. Suitable plant promoters are, in principle, all of the above-described promoters.

The nucleic acid sequences present in the expression cassettes according to the invention can be linked operably to further genetic control sequences in addition to a promoter. The term "genetic control sequences" is to be understood in the broad sense and refers to all those sequences which have an effect on the generation or the function of the expression cassette according to the invention. For example, genetic control sequences modify the transcription and translation in prokaryotic or eukaryotic organisms. Preferably, the expression cassette according to the invention comprise the promoter with specificity for the embryonal epidermis and/or the flower 5'-upstream of the nucleic acid sequence in question to be expressed recombinantly, and 3'-downstream a terminator sequence as additional genetic control sequence and, if appropriate, further customary regulatory elements, in each case linked functionally to the nucleic acid sequence to be expressed recombinantly.

Genetic control sequences also comprise further promoters, promoter elements or minimal promoters, all of which can modify the expression-governing properties. Thus, for example, the tissue-specific expression may additionally depend on certain stress factors, owing to genetic control sequences. Such elements have been described, for example, for water stress, abscisic acid (Lam E and Chua NH (1991) J Biol Chem 266(26): 17131-17135) and heat stress (Schoffl F et al. (1989) Mol Gen Genetics 217(2-3):246-53).

In principle, all natural promoters together with their regulatory sequences such as those mentioned above may be used for the method according to the invention. In addition, synthetic promoters may also be used advantageously.

Genetic control sequences furthermore also comprise the 5'-untranslated regions, introns or noncoding 3'-region of genes, such as, for example, the actin-1 intron, or the Adh1-S introns

1, 2 and 6 (general reference: The Maize Handbook, Chapter 116, Freeling and Walbot, Eds., Springer, New York (1994)). It has been demonstrated that they may play a significant role in the regulation of gene expression. Thus, it has been demonstrated that 5'-untranslated sequences can enhance the transient expression of heterologous genes. Examples of translation enhancers which may be mentioned are the tobacco mosaic virus 5' leader sequence (Gallie et al. (1987) Nucl Acids Res 15:8693-8711) and the like. Furthermore, they may promote tissue specificity (Rouster J et al. (1998) Plant J 15:435-440).

The expression cassette may advantageously comprise one or more of what are known as enhancer sequences, linked functionally to the promoter, which make possible an increased recombinant expression of the nucleic acid sequence. Additional advantageous sequences, such as further regulatory elements or terminators, may also be inserted at the 3' end of the nucleic acid sequences to be expressed recombinantly. One or more copies of the nucleic acid sequences to be expressed recombinantly may be present in the gene construct.

Polyadenylation signals which are suitable as control sequences are plant polyadenylation signals, preferably those which essentially correspond to T-DNA polyadenylation signals from *Agrobacterium tumefaciens*, in particular to gene 3 of the T-DNA (octopine synthase) of the Ti plasmid pTiACHS (Gielen et al. (1984) EMBO J 3:835 et seq.) or functional equivalents thereof. Examples of terminator sequences which are especially suitable are the OCS (octopine synthase) terminator and the NOS (nopaline synthase) terminator.

Control sequences are furthermore to be understood as those which make possible homologous recombination or insertion into the genome of a host organism or which permit removal from the genome. Upon homologous recombination, for example the natural promoter of a particular gene can be exchanged to a promoter with specificity for the embryonal epidermis and/or the flower. Methods such as the cre/lox technology permit a tissue-specific, if appropriate inducible, removal of the expression cassette from the genome of the host organism (Sauer B (1998) Methods. 14(4):381-92). Here, certain flanking sequences (lox sequences), which later make possible a removal by means of the cre recombinase, are added to the target gene.

An expression cassette and vectors derived therefrom may comprise further functional elements. The term functional element is to be understood in the broad sense and refers to all those elements

which have an effect on the generation, amplification or function of the expression cassettes, vectors or transgenic organisms according to the invention. The following may be mentioned by way of example, but not by limitation:

a) Selection markers which confer a resistance to metabolism inhibitors (such as 2-deoxyglucose-6-phosphate (WO 98/45456)), antibiotics or biocides, preferably herbicides, such as, for example, kanamycin, G 418, bleomycin, hygromycin or phosphinothricin etc. Especially preferred selection markers are those which confer resistance to herbicides. Examples which may be mentioned are: DNA sequences which encode phosphinothricin acetyl transferases (PAT) and which inactivate glutamin synthase inhibitors (bar and pat genes), 5-enolpyruvylshikimate-3-phosphate synthase genes (EPSP synthase genes), which confer resistance to Glyphosate® (N-(phosphonomethyl)glycine), the gox gene, which encodes Glyphosate®-degrading enzymes (glyphosate oxidoreductase), the deh gene (encoding a dehalogenase which inactivates Dalapon), sulfonylurea- and imidazolinone-inactivating acetolactate synthases, and bxn genes, which encode bromoxynil-degrading nitrilase enzymes, the aasa gene, which confers resistance to the antibiotic apectinomycin, the streptomycin phosphotransferase (spt) gene, which allows resistance to streptomycin, the neomycin phosphotransferase (nptII) gene, which confers resistance to kanamycin or geneticin, the hygromycin phosphotransferase (hpt) gene, which mediates resistance to hygromycin, the acetolactate synthase gene (ALS), which confers resistance to sulfonylurea herbicides (for example mutated ALS variants with, for example, the S4 and/or Hra mutation).

b) Reporter genes which encode readily quantifiable proteins and, via their color or enzyme activity, make possible an assessment of the transformation efficacy, the site of expression or the time of expression. Very especially preferred in this context are reporter proteins (Schenborn E, Groskreutz D. Mol Biotechnol. 1999; 13(1):29-44) such as the green fluorescent protein (GFP) (Sheen et al. (1995) Plant Journal 8(5):777-784; Haseloff et al. (1997) Proc Natl Acad Sci USA 94(6):2122-2127; Reichel et al. (1996) Proc Natl Acad Sci USA 93(12):5888-5893; Tian et al. (1997) Plant Cell Rep 16:267-271; WO 97/41228; Chui WL et al. (1996) Curr Biol 6:325-330; Leffel SM et al. (1997) Biotechniques. 23(5):912-8), chloramphenicol transferase, a luciferase (Ow et al. (1986) Science 234:856-859; Millar et al. (1992) Plant Mol Biol Rep 10:324-414), the aequoringen (Prasher et al.

(1985) Biochem Biophys Res Commun 126(3):1259-1268), β-galactosidase, R-locus gene (encode a protein which regulates the production of anthocyanin pigments (red coloration) in plant tissue and thus makes possible a direct analysis of the promoter activity without addition of extra adjuvants or chromogenic substrates; Dellaporta et al., In: Chromosome Structure and Function: Impact of New Concepts, 18th Stadler Genetics Symposium, 11:263-282, 1988), with β-glucuronidase being very especially preferred (Jefferson et al., EMBO J. 1987, 6, 3901-3907).

c) Origins of replication, which ensure amplification of the expression cassettes or vectors according to the invention in, for example, E. coli. Examples which may be mentioned are ORI (origin of DNA replication) the pBR322 ori or the P15A ori (Sambrook et al.: Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989).

d) Elements which are necessary for Agrobacterium-mediated plant transformation, such as, for example, the right or left border of the T-DNA or the vir region.

The introduction of an expression cassette according to the invention into an organism or cells, tissues, organs, parts or seeds thereof (preferably into plants or plant cells, tissue, organs, parts or seeds) can be effected advantageously using vectors which comprise the expression cassettes. The expression cassette can be introduced into the vector (for example a plasmid vector) via a suitable restriction cleavage site. The plasmid formed is first introduced into E. coli. Correctly transformed E. coli are selected, grown, and the recombinant plasmid is obtained by the methods familiar to the skilled worker. Restriction analysis and sequencing may serve to verify the cloning step.

Examples of vectors may be plasmids, cosmids, phages, viruses or else agrobacteria. In an advantageous embodiment, the expression cassette is introduced by means of plasmid vectors. Preferred vectors are those which make possible a stable integration of the expression cassette into the host genome.

The generation of a transformed organism (or of a transformed cell or tissue) requires that the DNA, RNA or protein in question is introduced into the corresponding host cell.

A multiplicity of methods are available for this procedure, which is termed transformation (or transduction or transfection) (Keown et al. (1990) Methods in Enzymology 185:527-537). For example, the DNA or RNA can be introduced directly by microinjection or by bombardment with DNA-coated microparticles. Also, the cell can be permeabilized chemically, for example using polyethylene glycol, so that the DNA can enter the cell by diffusion. The DNA can also be introduced by protoplast fusion with other DNA-containing units such as minicells, cells, lysosomes or liposomes. Another suitable method of introducing DNA is electroporation, where the cells are permeabilized reversibly by an electrical pulse. Suitable methods have been described (for example by Bilang et al. (1991) Gene 100:247-250; Scheid et al. (1991) Mol Gen Genet 228:104-112; Guerche et al. (1987) Plant Science 52:111-116; Neuhause et al. (1987) Theor Appl Genet 75:30-36; Klein et al. (1987) Nature 327:70-73; Howell et al. (1980) Science 208:1265; Horsch et al.(1985) Science 227:1229-1231; DeBlock et al. (1989) Plant Physiology 91:694-701; Methods for Plant Molecular Biology (Weissbach and Weissbach, eds.) Academic Press Inc. (1988); and Methods in Plant Molecular Biology (Schuler and Zielinski, eds.) Academic Press Inc. (1989)).

In plants, the above-described methods of transforming and regenerating plants from plant tissues or plant cells are exploited for transient or stable transformation. Suitable methods are especially protoplast transformation by polyethylene-glycol-induced DNA uptake, the biolistic method with the gene gun, what is known as the particle bombardment method, electroporation, incubation of dry embryos in DNA-containing solution, and microinjection.

In addition to these "direct" transformation techniques, transformation can also be effected by bacterial infection by means of Agrobacterium tumefaciens or Agrobacterium rhizogenes. The Agrobacterium-mediated transformation is best suited to dicotyledonous plant cells. The methods are described, for example, by Horsch RB et al. (1985) Science 225: 1229f.

When agrobacteria are used, the expression cassette must be integrated into specific plasmids, either into a shuttle or intermediate vector, or into a binary vector. If a Ti or Ri plasmid is to be used for the transformation, at least the right border, but in most cases the right and left border, of the Ti or Ri plasmid T-DNA is linked to the transgenic expression construct to be introduced in the form of a flanking region.

Binary vectors are preferably used. Binary vectors are capable of replication both in E. coli and in Agrobacterium. As a rule, they comprise a selection marker gene for the selection of transformed plants (see above) and a linker or polylinker flanked by the right and left T-DNA border sequence. They can be transformed directly into Agrobacterium (Holsters et al. (1978) Mol Gen Genet 163:181-187). Apart from the T-DNA region, they can additionally comprise elements such as a selection marker gene for the selection of transformed E. coli or agrobacteria (e.g. the nptIII gene). The Agrobacterium which acts as host organism in this case should already contain a plasmid with the vir region. The latter is required for transferring the T-DNA to the plant cell. An Agrobacterium transformed in this way can be used for transforming plant cells. The use of T-DNA for transforming plant cells has been studied and described intensively (EP 120 516; Hoekema, In: The Binary Plant Vector System, Offsetdrukkerij Kanters B.V., Alblasserdam, Chapter V; An et al. (1985) EMBO J 4:277-287). Various binary vectors are known, some of which are commercially available such as, for example, pBI101.2 or pBIN19 (Clontech Laboratories, Inc. USA).

Further promoters which are suitable for expression in plants are described (Rogers et al. (1987) Meth in Enzymol 153:253-277; Schardl et al. (1987) Gene 61:1-11; Berger et al. (1989) Proc Natl Acad Sci USA 86:8402-8406).

Direct transformation techniques are suitable for any organism and cell type.

The plasmid used need not meet any particular requirements in the case of the injection or electroporation of DNA or RNA into plant cells. Simple plasmids such as those of the pUC series can be used. If complete plants are to be regenerated from the transformed cells, it is advantageous for an additional selectable marker gene to be located on the plasmid.

Stably transformed cells, i.e. those which contain the introduced DNA integrated into the DNA of the host cell, can be selected from untransformed cells when a selectable marker is part of the DNA introduced. Examples of genes which can act as markers are all those which are capable of conferring resistance to a biocide (for example an antibiotic, herbicide or a metabolism inhibitor such as 2-deoxyglucose-6-phosphate WO 98/45456) (see above). Transformed cells which express such marker genes are capable of surviving in the presence of concentrations of a corresponding antibiotic or herbicide which kill an untransformed wild type. Examples are mentioned above and preferably comprise the bar

gene, which confers resistance to the herbicide phosphinothricin (Rathore KS et al. (1993) Plant Mol Biol 21(5):871-884), the nptII gene, which confers resistance to kanamycin, the hpt gene, which confers resistance to hygromycin, or the EPSP gene, which confers resistance to the herbicide glyphosate. The selection marker permits the selection of transformed cells from untransformed cells (McCormick et al. (1986) Plant Cell Reports 5:81-84). The resulting plants can be bred and hybridized in the customary fashion. Two or more generations should be grown in order to ensure that the genomic integration is stable and hereditary.

The abovementioned methods are described, for example, in Jenes B et al.(1993) Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, edited by SD Kung and R Wu, Academic Press, pp. 128-143 and in Potrykus (1991) Annu Rev Plant Physiol Plant Molec Biol 42:205-225. The construct to be expressed is preferably cloned into a vector which is suitable for the transformation of Agrobacterium tumefaciens, for example pBin19 (Bevan et al. (1984) Nucl Acids Res 12:8711f).

As soon as a transformed plant cell has been generated, a complete plant can be obtained using methods known to the skilled worker. For example, callus cultures are used as starting material. The development of shoot and root can be induced from this as yet undifferentiated cell biomass in a known fashion. The shoots obtained can be planted out and bred.

The skilled worker is familiar with such methods of regenerating intact plants from plant cells and plant parts. Methods to do so are described, for example, by Fennell et al. (1992) Plant Cell Rep. 11: 567-570; Stoeger et al (1995) Plant Cell Rep. 14:273-278; Jahne et al. (1994) Theor Appl Genet 89:525-533.

The method according to the invention can advantageously be combined with further methods which bring about a pathogen resistance (for example against insects, fungi, bacteria, nematodes and the like), stress resistance or another improvement of the plant's properties. Examples are mentioned in Dunwell JM, Transgenic approaches to crop improvement, J Exp Bot. 2000;51 Spec No; pages 487-96, inter alia.

With regard to, for example, a nucleic acid sequence, an expression cassette or a vector comprising said nucleic acid sequence or an organism transformed with said nucleic acid sequence, expression cassette or vector, "transgenic" means all those

constructs which have been generated by recombinant methods in which either

a) the NADPH oxidase nucleic acid sequence, or

b) a genetic control sequence which is functionally linked with the NADPH oxidase nucleic acid sequence, for example a promoter, or

c) (a) and (b)

are not located in their natural genetic environment or have been modified by recombinant methods, an example of a modification being a substitution, addition, deletion, inversion or insertion of one or more nucleotide residues. Natural genetic environment refers to the natural chromosomal locus in the source organism, or to the presence in a genomic library. In the case of a genomic library, the natural genetic environment of the nucleic acid sequence is preferably at least partially retained. The environment flanks the nucleic acid sequence at at least one side and has a sequence length of at least 50 bp, preferably at least 500 bp, especially preferably at least 1000 bp, very especially preferably at least 5000 bp. A naturally occurring expression cassette - for example the naturally occurring combination of the NADPH oxidase promoter and the corresponding NADPH oxidase gene - becomes a transgenic expression cassette when the latter is modified by nonnatural, synthetic ("artificial") methods such as, for example, mutagenization. Suitable methods are described (US 5,565,350; WO 00/15815; see also above).

Another aspect of the invention relates to transgenic organisms transformed with at least one nucleic acid sequence, expression cassette or vector according to the invention, and to cells, cell cultures, tissues, parts - such as, for example in the case of plant organisms, leaves, roots and the like - or propagation material derived from such organisms. Organism is to be understood in the broad sense and means prokaryotic and eukaryotic organisms, preferably bacteria, yeasts, fungi, animal and plant organisms.

The following are preferred:

a) fungi such as Aspergillus, Eremothecium, Trichoderma, Ashbya, Neurospora, Fusarium, Beauveria or further fungi described in Indian Chem Eng. Section B. Vol 37, No 1,2 (1995) on page 15,

table 6. Especially preferred is the filamentous Hemiascomycete Ashbya gossypii or Eremothecium ashbyii,

b) yeasts such as Candida, Saccharomyces, Hansenula or Pichia, with Saccharomyces cerevisiae or Pichia pastoris (ATCC Accession No. 201178) being especially preferred,

c) plants in accordance with the abovementioned definition for "plants",

d) vertebrates and invertebrates. Especially preferred vertebrates are nonhuman mammals such as dogs, cats, sheep, goats, chickens, mice, rats, cattle or horses. Preferred animal cells comprise CHO, COS, HEK293 cells. Preferred invertebrates comprise insect cells such as Drosophila S2 and Spodoptera Sf9 or Sf21 cells,

e) prokaryotic organisms such as Gram-positive or Gram-negative bacteria such as Acetobacter, Gluconobacter, Corynebacterium, Brevibacterium, Bacillus, Clostridium, Cyanobacter, Escherichia (especially Escherichia coli), Serratia, Staphylococcus, Aerobacter, Alcaligenes, Penicillium or Klebsiella.

Host or starting organisms which are preferred as transgenic organisms are especially plants in accordance with the abovementioned definition. Included within the scope of the invention are all genera and species of higher and lower plants of the Plant Kingdom. Furthermore included are the mature plants, seeds, shoots and seedlings, and parts, propagation materials and culture derived from them, for example cell cultures. Mature plants means plants at any developmental stage beyond the seedling stage. Seedling means a young immature plant in an early developmental stage. Plants which are especially preferred as host organisms are plants to which the method according to the invention for obtaining a pathogen resistance in accordance with the abovementioned criteria can be applied. Very especially preferred are monocotyledonous plants such as wheat, oats, millet, barley, rye, maize, rice, buckwheat, sorghum, triticale, spelt, linseed, sugar cane, and dicotyledonous crop plants such as oilseed rape, canola, cress, Arabidopsis, cabbages, soybeans, alfalfa, pea, bean plants, peanut, potato, tobacco, tomato, egg plant, capsicum, sunflower, tagetes, lettuce, Calendula, melon, pumpkin/squash or zucchini.

The transgenic organisms can be generated with the above-described methods for the transformation or transfection of organisms.

A further aspect of the invention relates to the use of transgenic organisms according to the invention and of the cells, cell cultures, parts - such as for example in the case of transgenic plant organisms roots, leaves and the like - and transgenic propagation material such as seeds or fruits derived from these organisms for the production of foodstuffs, feedstuffs, pharmaceuticals or fine chemicals.

Furthermore preferred is a method for the recombinant production of pharmaceuticals or fine chemicals in host organisms, where a host organism is transformed with one of the above-described expression cassettes and this expression cassette comprises one or more structural genes which encode the desired fine chemical or catalyze the biosynthesis of the desired fine chemical, the transformed host organism is cultured, and the desired fine chemical is isolated from the culture medium. This method can be applied widely for fine chemicals such as enzymes, vitamins, amino acids, sugars, fatty acids, natural and synthetic flavorings, aroma substances and colorants. Especially preferred is the production of tocopherols and tocotrienols and of carotenoids. Culturing the transformed host organisms, and the isolation from the host organisms or from the culture medium, are carried out with methods known to the skilled worker. The production of pharmaceuticals, such as, for example, antibodies or vaccines, is described by Hood EE, Jilka JM (1999) Curr Opin Biotechnol 10(4):382-6; Ma JK, Vine ND (1999) Curr Top Microbiol Immunol 236:275-92.

Sequences

1. SEQ ID NO: 1 nucleic acid sequence encoding a barley (Hordeum vulgare) NADPH oxidase.

2. SEQ ID NO: 2 amino acid sequence encoding a barley (Hordeum vulgare) NADPH oxidase.

3. SEQ ID NO: 3 nucleic acid sequence encoding a rice (Oryza sativa var. japonica) NADPH oxidase

4. SEQ ID NO: 4 amino acid sequence encoding a rice (Oryza sativa var. japonica) NADPH oxidase

5. SEQ ID NO: 5 nucleic acid sequence encoding a Nicotiana tabacum NADPH oxidase

6. SEQ ID NO: 6 amino acid sequence encoding a Nicotiana tabacum NADPH oxidase

7. SEQ ID NO: 7 nucleic acid sequence encoding a potato (Solanum tuberosum) NADPH oxidase

8. SEQ ID NO: 8 amino acid sequence encoding a potato (Solanum tuberosum) NADPH oxidase

9. SEQ ID NO: 9 nucleic acid sequence encoding a tomato (Lycopersicon esculentum) NADPH oxidase

10. SEQ ID NO: 10 amino acid sequence encoding a tomato (Lycopersicon esculentum) NADPH oxidase

11. SEQ ID NO: 11 nucleic acid sequence encoding a NADPH oxidase aus Arabidopsis thaliana (RbohF)

12. SEQ ID NO: 12 amino acid sequence encoding a NADPH oxidase aus NADPH oxidase Arabidopsis thaliana (RbohF)

13. SEQ ID NO: 13 nucleic acid sequence encoding an Arabidopsis thaliana (RbohD) NADPH oxidase

14. SEQ ID NO: 14 amino acid sequence encoding an Arabidopsis thaliana (RbohD) NADPH oxidase

15. SEQ ID NO: 15 nucleic acid sequence encoding a Nicotiana tabacum (rboh) NADPH oxidase

16. SEQ ID NO: 16 amino acid sequence encoding a Nicotiana tabacum (rboh) NADPH oxidase

17. SEQ ID NO: 17 nucleic acid sequence encoding a rice (Oryza sativa var. japonica) NADPH oxidase

18. SEQ ID NO: 18 amino acid sequence encoding a rice (Oryza sativa var. japonica) NADPH oxidase

19. SEQ ID NO: 19 nucleic acid sequence encoding an Arabidopsis thaliana (RbohC) NADPH oxidase

20. SEQ ID NO: 20 amino acid sequence encoding an Arabidopsis thaliana (RbohC) NADPH oxidase

21. SEQ ID NO: 21 nucleic acid sequence encoding an Arabidopsis thaliana (RbohA) NADPH oxidase

22. SEQ ID NO: 22 amino acid sequence encoding an Arabidopsis thaliana (RbohA) NADPH oxidase

23. SEQ ID NO: 23 oligonucleotide primer 5' NAOX 5'-GARCAAGGCTCTTTTGATTG-3'

24. SEQ ID NO: 24 oligonucleotide primer 3' Naox 5'-GAAATGCTCCTTATGGAATTC-3'

Figures

Fig. 1: RNA interference with *pNAox*-dsRNA reduces the penetration efficiency of powdery mildew of barley BghA6 in barley.

The relative penetration efficiency (RPE) was determined in five individual experiments with inoculation with *Bgh* from barley cv Pallas. The RPE is calculated as the difference between the penetration efficiency of pNAox-dsRNA-transformed cells and the penetration efficiency of control-dsRNA-transformed cells (here: average penetration efficiency 38.74%). The percent RPE (% RPE) is calculated from the RPE minus 1, multiplied by 100.

$$\text{RPE} = \frac{\text{[PE in pNAox-dsRNA-transformed cells]}}{\text{[PE in control-dsRNA transformed cells]}}$$

$$\%\ \text{RPE} = 100 * (\text{RPE}-1)$$

The columns (1) to (5) represent the % RPE (i.e. the deviation of the penetration efficiency from the average of the penetration efficiency of the control) when evaluating at least 100 interac-

tion sites for in each case one independent experiment. The column (m) represents the average % RPE of the experiments. The error bar indicates the standard error.

"Control dsRNA" represents the parallel experiments with a control dsRNA. "pNAox" dsRNA represents the experiments with the dsRNA of the barley NADPH oxidase.

In cells which have been bombarded with pNAox-dsRNA, the % RPE was markedly (significance p=0.0054) reduced in comparison with cells bombarded with a control dsRNA (*TR*: human thyroid receptor dsRNA).

Examples

General methods:

The chemical synthesis of oligonucleotides can be effected for example in the known manner by the phosphoamidite method (Voet, Voet, 2nd edition, Wiley Press New York, pages 896-897). The cloning steps carried out within the scope of the present invention, such as, for example, restriction cleavages, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linking DNA fragments, transformation of E. coli cells, bacterial cultures, phage multiplication and sequence analysis of recombinant DNA, are carried out as described by Sambrook et al. (1989) Cold Spring Harbor Laboratory Press; ISBN 0-87969-309-6. Recombinant DNA molecules are sequenced with a laser fluorescence DNA sequencer from MWG Licor following the method of Sanger (Sanger et al. (1977) Proc Natl Acad Sci USA 74:5463-5467).

Example 1: Plants, pathogens and inoculation

The variety Pallas was provided by Lisa Munk, Department of Plant Pathology, Royal Veterinary and Agricultural University, Copenhagen, Denmark. Its production is described (Kølster P et al. (1986) Crop Sci 26: 903-907).

Unless otherwise described, the seed, which had been pregerminated for 12 to 36 hours in the dark on damp filter paper, was placed at a rate of 5 kernels along the edge of a square pot (8 × 8 cm) in Fruhstorfer soil, type P, covered with soil and watered regularly with tap water. All plants were grown in controlled-environment cabinets or chambers at 16 to 18°C, 50 to 60% relative atmospheric humidity and a 16-hour-light/8-hour-dark photoperiod at 3000 or 5000 lux (photon flux density 50 or

60 µmols^{-1}m^{-2}) for 5 to 8 days and used in the experiments during the seedling stage. In experiments in which primary leaves were treated, the latter were fully developed.

Prior to carrying out the transient transfection experiments, the plants were grown in controlled environment cabinets or chambers at 24°C daytime temperature, 20°C nighttime temperature, 50 to 60% relative atmospheric humidity and a 16-hour-light/8-hour-dark photoperiod at 30 000 lux.

Powdery mildew of barley *Blumeria graminis* (DC) Speer f.sp. *hordei* Em. Marchal race A6 (Wiberg A (1974) Hereditas 77: 89-148) (BghA6) was used for the inoculation of barley plants. The fungus was provided from the Department of Biometry, JLU Gießen. The inoculum was propagated in controlled-environment chambers under identical conditions as described above for the plants by transferring the conidia of infected material to regularly grown 7-day-old barley plants cv. Golden Promise at a density of 100 conidia/mm^2.

The inoculation with BghA6 was carried out using 7-day-old seedlings by shaking off the conidia from infected plants in an inoculation tower at approximately 100 conidia/mm^2 (unless otherwise specified).

Example 2: Cloning of the barley pNAox cDNA sequence

The cDNA fragments required for the isolation of the HvpNAox cDNA, its cloning, sequencing and generation of probes were obtained by RT-PCR using the "One Step RT-PCR Kit" (Life Technologies, Karlsruhe, Germany, or Qiagen, Hilden, Germany). To this end, a total RNA from barley seedlings was used as template. The RNA was isolated from Pallas 3, 5 and 7 days after germination. In addition, RNA was isolated from Pallas and from the backcrossed lines with *mlo5*, *Mlg* or *Mla12* 1, 2 and 5 days after inoculation with *Bgh*A6 on day 7 after germination. The RT-PCR was carried out using primers which are derived from conserved regions of the gp91phox homologs from rice and Arabidopsis thaliana (GenBank Acc. No.: X93301 and AB008111):

5' NAOX: 5'-GARCAAGGCTCTTTTGATTG-3' (SEQ ID NO: 23) and

3' Naox: 5' GAAATGCTCCTTATGGAATTC 3'(SEQ ID NO: 24)

In each case 1000 ng of total DNA, 0.4 mM dNTPs, in each case 0.6 mM OPN-1 and OPN-2 primer, 10 µl of RNase inhibitor and 1 µl of enzyme mix in 1x RT buffer (*one step RT-PCR Kit*, Qiagen, Hilden) were employed for the reaction.

The following temperature program is used (PTC-100TM model 96V; MJ Research, Inc., Watertown, Massachussetts):

1	cycle of 30 minutes at 50°C
1	cycle of 150 seconds at 94°C
30	cycles of 94°C for 45 seconds, 55°C for 1 minute and 72°C for 2 minutes
1	cycle of 72°C for 7 minutes

The PCR products were separated by means of 2% w/v agarose gel electrophoresis. This gave a 378 bp RT-PCR product (SEQ ID NO: 1) which encodes a part of the open reading frame of the barley NADPH oxidase. The corresponding cDNA was isolated from an agarose gel and cloned in the pGEM-T vector (Promega, Mannheim, Germany) by means of T-overhang ligation. The cDNAs were sequenced starting from the plasmid DNA using the "Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit" (Amersham, Freiburg, Germany). The construct was named pGEM-T-pNAox.

Example 3: In-vitro synthesis of the pNAox dsRNA

The plasmid, which had been employed for the in-vitro RNA transcription, comprises the T7 and SP6 promoters at the respective ends of the inserted nucleic acid sequence, which makes possible the synthesis of sense RNA and antisense RNA. The plasmid can be linearized with suitable restriction enzymes (ApaI for SP6 polymerase and PstI for T7 polymerase) in order to ensure correct transcription of the inserted nucleic acid sequence and to prevent read-through into vectorial sequences. To this end, in each case 10 µg of pGEM-T-pNAox plasmid DNA were cut with ApaI for SP6 polymerase and with and PstI for T7 polymerase. The cut plasmids are extracted in 200 µl of water with the same volume phenol/chloroform/isoamyl alcohol, transferred into a fresh Eppendorf vessel (RNAse-free) and centrifuged for 5 minutes at 20 000 g. 180 µl of the plasmid solution were treated with 420 µl of ethanol, placed on ice and subsequently precipitated by centrifugation for 30 minutes at 20 000 g and -4°C. The precipitate was taken up in 10 µl of TE buffer. The preparations in question were employed directly in an in-vitro transcription with T7-RNA polymerase and with SP6-RNA polymerase, respectively. RNA polymerases were obtaied from Roche Molecular Biology, Mannheim, Germany.

Each transcription mixture contained the following in a volume of 40 µl:

2 µl linearized plasmid DNA (1 ∝g)
2 µl NTPs (25 mM) (1.25 mM of each NTP)
4 µl 10x reaction buffer (Roche Molecular Biology),
1 µl RNAsin RNAsin (27 units; Roche Molecular Biology),
2 µl RNA polymerase (40 units)
29 µl DEPC water

After 2 hours of incubation at 37°C, in each case some of the reaction mixtures from the transcription of the sense and antisense strands were mixed, denatured for 5 minutes at 95°C and thereafter hybridized with one another (annealed) by cooling over 30 minutes to a final temperature of 37°C. As an alternative, the mixture of sense and antisense strand can also be cooled for 30 minutes at -20°C after the denaturation. The protein precipitate which formed during denaturation and hybridization was removed by briefly centrifuging at 20 800 g, and the supernatant was used directly for coating tungsten particles (see hereinbelow). For the analysis, in each case 1 µl of each RNA strand and of the dsRNA were separated on a non-denaturing agarose gel. Successful hybridization is evident by a band shift towards higher molecular weight in comparison with the individual strands.

4 µl of the dsRNA were precipitated with ethanol (by addition of 6 µl of water, 1 µl of 3M sodium acetate solution and 25 µl of ethanol, and centrifugation for at least 5 minutes at 20 000 g and 4°C) and resuspended in 500 µl of water. The absorption spectrum between 230 and 300 nm was measured or the absorption at 280 and 260 nm was determined to determine the purity and the concentration of the dsRNA. As a rule, 80 to 100 µg of dsRNA with an OD_{260}/OD_{280} ratio of 1.80 to 1.95 were obtained. If desired, a digestion with DNase I may be carried out, but this has no substantial effect on subsequent results.

The dsRNA of the human thyroid receptor (starting vector pT7beta-Sal (Norman C et al. (1988) Cell 55(6):989-1003), provided by Dr. Baniahmad, Department of Genetics, Gießen, Germany; the sequence of the insert is described under the GenBank Acc. No.: NM_000461) acted as control dsRNA. The plasmid was digested with PvuII to generate the sense RNA and with HindIII to generate the antisense RNA, and the RNA was then transcribed using T7 or SP6 RNA polymerase. The individual process steps for the generation of the control dsRNA are carried out analogously to those described above for the pNAox-dsRNA.

Example 4: Transient transformation, RNAi and evaluation of the development of the fungal pathogen

Barley cv Pallas leaf segments were transformed with a *pNAox* dsRNA together with a GFP expression vector. Thereafer the leaves were inoculated with Bgh and the result was analyzed after 48 h by means of light and fluorescence microscopy. The penetration into GFP-expressing cells was assessed by detecting haustoria in live cells and by assessing the fungal development in precisely those cells. In all five experiments, the bombardment of barley cv Pallas with *pNAox* dsRNA resulted in a reduced number of cells which were successfully penetrated by Bgh in comparison with cells which had been bombarded with foreign control dsRNA (human thyroid hormone receptor dsRNA, TR). The resistance-inducing effect of the *pNAox* dsRNA resulted in an average reduction of the Bgh penetration efficiency by 35% (Fig. 4).

A method which had already been described for the biolistic introduction of dsRNA into epidermal cells of barley leaves was employed for the transient transformation (Schweizer P et al. (1999) Mol Plant Microbe Interact 12:647-54; Schweizer P et al. (2000) Plant J 2000 24: 895-903). Tungsten particles 1.1 µm in diameter (particle density 25 mg/ml) were coated with dsRNA (preparation see above) together with plasmid DNA of the vector *pGFP* (GFP under the control of the CaMV 35S promoters) as transformation marker. To this end, the following amounts of dsRNA and reporter plasmid were used for the coating per shot: 1 µg pGFP and 2 µg dsRNA. Double-stranded RNA was synthesized by annealing sense and antisense RNA *in vitro* (see above).

To prepare microcarriers, 55 mg of tungsten particles (M 17, diameter 1.1 µm; Bio-Rad, Munich) were washed twice with 1 ml of autoclave-distilled water and once with 1 ml of absolute ethanol, dried and taken up in 1 ml of 50% strength glycerol (approximately 50 mg/ml stock solution). The solution was diluted with 50% glycerol to 25 mg/ml, mixed thoroughly prior to use and suspended in an ultrasonic bath. To coat microcarriers, 1 µg of plasmid, 2 µg of dsRNA (1 µl), 12.5 µl of tungsten particle suspension (25 mg/ml), 12.5 µl of 1 M $Ca(NO_3)_2$ solution (pH 10) per shot were combined dropwise with constant mixing, left to stand for 10 minutes at RT, centrifuged briefly, and 20 µl of the supernatant were removed. The remainder with the tungsten particles is resuspended (ultrasonic bath) and employed in the experiment.

Barley primary leaf segments approximately 4 cm in length were used. The tissues were placed on 0.5% Phytagar (GibcoBRL™ Life Technologies™, Karlsruhe) supplemented with 20 µg/ml benzimida-

zole in Petri dishes (diameter 6.5 cm) and, immediately before the particle bombardment, the edges were covered with a stencil with a rectangular opening of dimensions 2.2 cm × 2.3 cm. One after the other, the dishes were placed on the bottom of the vacuum chamber (Schweizer P et al. (1999) Mol Plant Microbe Interact 12:647-54) over which a nylon mesh (mesh size 0.2 mm, Millipore, Eschborn) had been inserted on a perforated sheet to act as diffusor (5 cm above the bottom, 11 cm underneath the macrocarriers, see hereinbelow) in order to diffuse particle clumps and to slow down the particle stream. The macrocarrier attached at the top of the chamber (plastic sterile filter holder, 13 mm, Gelman Sciences, Swinney, UK) was loaded with 5.8 µl of DNA-coated tungsten particles per shot (microcarriers, see hereinbelow). Using a diaphragm vacuum pump (Vacuubrand, Wertheim), the pressure in the chamber was reduced by 0.9 bar, and the tungsten particles were fired at the surface of the plant tissue at a helium-gas pressure of 9 bar. Immediately thereafter, the chamber was aerated. To label transformed cells, the leaves were bombarded with the plasmid (pGFP; vector on pUC18-basis, CaMV 35S promoter/terminator cassette with inserted GFP gene; Schweizer P et al. (1999) Mol Plant Microbe Interact 12:647-54; provided by Dr. P. Schweizer, Department of Plant Genetics IPK, Gatersleben, Germany). Each time a different plasmid was used for the bombardments, the macrocarrier was cleaned thoroughly with water beforehand. After incubation for four hours after the bombardment with slightly open Petri dishes at RT and with daylight, the leaves were incubated with 100 conidia/mm^2 of powdery mildew of barley (race A6) and incubated under identical conditions for a further 40 to 48 hours.

Leaf segments were bombarded with the coated particles using a particle inflow gun. For each shot, 312 µg of tungsten particles were applied. 4 hours after the bombardment, the leaves were inoculated with *Blumeria graminis f.sp. hordei* mildew (race A6) and, after a further 40 hours, evaluated for symptoms of infection. The result (for example the penetration efficiency, defined as percentage of attacked cells with a mature haustorium and a secondary elongating hypha were analyzed by means of fluorescence and light microscopy. An inoculation with 100 conidia/mm^2 results in an infection frequency of approximately 50% of the transformed cells. A minimum of 100 interaction sites was evaluated for each individual experiment. Transformed (GFP-expressing) cells were identified under excitation with blue light. Three different categories of transformed cells were distinguished:

1. Penetrated cells containing a readily recognizable haustorium. A cell with more than one haustorium was considered as one cell.

2. Cells which, while attacked by a fungal appressorium, contain no haustorium. A cell which has been attacked more than once by Bgh, but which contains no haustorium, was considered as one cell.

3. Cells which are not infected by Bgh.

Stomatal cells and guard cells were excluded from the assessment. Surface structures of Bgh were analyzed by means of light microscopy or fluorescence staining of the fungus with 0.1% Calcofluor (w/v in water) for 30 seconds. The fungal development can be evaluated readily by fluorescence microscopy following staining with Calcofluor. In pNAox-dsRNA-transformed cells, the fungus develops a primary and apressorial germ tube, but no haustorium. The development of a haustorium is a condition for the development of a secondary hypha.

The relative penetration efficiency (RPE) is calculated as the difference between the penetration efficiency of transformed cells (transformation with pNAox or control dsRNA) and the penetration efficiency of untransformed cells (here: average penetration efficiency 38.74%). The percent RPE (% RPE) is calculated from the RPE minus 1, multiplied by 100.

$$RPE = \frac{[PE \text{ in pNAox-dsRNA-transformed cells}]}{[PE \text{ in control-dsRNA transformed cells}]}$$

$$\% \text{ RPE} = 100 * (RPE-1)$$

The % RPE value (deviation of the average penetration efficiency of the control) is used to determine the susceptibility of cells transfected with pNAox-dsRNA (fig. 4).

In the case of the control dsRNA, five different experiments reveal no difference between the transfection with the control dsRNA and water with regard to the penetration efficiency of *Bgh*.

To rule out an effect of the dsRNA and the transformation rate or survival rate of the attacked cells, the number of GFP-expressing cells in control experiments and *pNAox*-dsRNA experiments was compared. The *pNAox*-dsRNA had no effect on the total number or the number of the attacked GFP-expressing cells.

Example 5: NADPH oxidase inhibition with diphenyleneiodonium chloride

The results were supported by further experiments with the NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI; table 1). In general, the experiments were carried out as described by Hückelhoven and Kogel, 1998.

Tab. 1: Effect of DPI on the defense against pathogens in Pallas[a]

	Interactions (% ± standard error)	
Type of interaction	Control[b]	200 µM DPI[c]
Penetration	68.25 ± 9.9	16.25 ± 0.5
Nonpenetration	24.25 ± 6.3	67.5 ± 9.5
HR (Hypersensitive response)	7.5 ± 3.7	16.25 ± 9.3

a The DPI treatment was carried out 12 hours after inoculation with the pathogen and the evaluation 36 hours after inoculation.

b Controll with 10 mM potassium phosphate buffer, pH 7.8, with DMSO content as in the DPI treatment.

c DPI dissolved in 10 mM potassium phosphate buffer, pH 7.8, starting from a 10 mg/ml DPI stock solution in DMSO.